Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 2593–2597, April 1993
Biochemistry

Molecular cloning and characterization of a cDNA showing negative regulation in v-*src*-transformed 3Y1 rat fibroblasts

(differential screening/zinc finger)

TOSHINORI OZAKI AND SHIGERU SAKIYAMA*

Division of Biochemistry, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuoh-ku, Chiba, 260, Japan

Communicated by John M. Buchanan, December 21, 1992

ABSTRACT **A differential screening procedure was used to isolate genes that are specifically down-regulated in transformed cells. After screening a cDNA library derived from normal rat fibroblast (3Y1) cells, we obtained several independent cDNAs whose mRNA level was substantially lower in 3Y1 cells transformed with Rous sarcoma virus than in untransformed 3Y1 cells. Among them, N03 cDNA has been characterized extensively. N03 mRNA was also absent from v-*mos*- or simian virus 40-transformed 3Y1 cells but was present in v-Ha-*ras*-transformed 3Y1 cells. Nucleotide sequence analysis showed that the N03 protein is composed of 178 amino acid residues and does not have any sequence similarities with proteins in the data base. A putative zinc-finger domain is located in the central part of the sequence and a proline-rich domain is in the C-terminal region. N03 mRNA was detected by Northern blot analysis in several tissues, including lung, kidney, intestine, and brain, but not in liver. Genomic Southern blot hybridization revealed that the N03 gene exists as a single copy in the rat genome and that closely related, single-copy genes are also present in chicken and human.**

Viral transformation results in a variety of phenotypic changes in the host cells, including decreased adhesiveness, rounder cell shape, and loss of cellular alignment. Although accumulated evidence suggests that the oncogene products are responsible for initiation and maintenance of these malignant phenotypes, the molecular basis of the mechanisms by which these oncogene products produce these changes is not well understood (1, 2).

The v-*src* gene, which is carried on the Rous sarcoma virus (RSV) genome, is one of the most characterized oncogenes. Its product ($pp60^{v\text{-}src}$) has a tyrosine-specific protein kinase activity and is associated with plasma membranes and cytoskeletal structure (3–7). To clarify the transformation mechanisms caused by RSV at the molecular level, several independent groups have identified and characterized cellular genes whose expression was increased (8–17) or decreased (18–20) upon viral transformation. However, the information regarding the relationship between RSV-induced changes in gene expression and the phenotypic alterations of the transformed cells is limited. Among the genes whose expression is altered upon transformation, those down-regulated seem to be more important, because this group might include candidate tumor-suppressor genes. Isolation of such genes was indeed reported in various systems (21, 22). Thus, we initiated a program using a differential screening procedure to obtain cDNAs that are expressed in normal rat fibroblast (3Y1) cells but not in 3Y1 cells transformed with RSV. In this report, we describe the molecular cloning and characterization of a cDNA whose expression is negatively regulated in v-*src*-transformed 3Y1 cells.†

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MATERIALS AND METHODS

Cells and Culture Conditions. The rat fibroblast line 3Y1 and the v-*src*-transformed 3Y1 line (v-*src*-3Y1) were gifts from H. Sakiyama, National Institute of Radiological Science, Chiba, Japan. The v-*mos*-transformed 3Y1, the simian virus 40 (SV40)-transformed 3Y1, and the v-Ha-*ras*-transformed 3Y1 cell lines were obtained from the Japanese Cancer Research Resources Bank. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Isolation of Total and Polyadenylylated RNA. Total RNA was extracted from culture cells by the SDS/phenol method. Poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)-cellulose (23).

Construction and Screening of the cDNA Library. Poly(A)$^+$ RNA derived from 3Y1 cells was used for the construction of the cDNA library. Double-stranded cDNAs were synthesized (24), methylated at *Eco*RI site(s), ligated to phosphorylated synthetic *Eco*RI linkers, and digested with excess *Eco*RI. The products were separated from free *Eco*RI linkers by Sephadex G-200 chromatography and inserted into the *Eco*RI site of λZAPII (Stratagene). After the phage DNA was packaged *in vitro*, the cDNA library was amplified once in *E. coli* XL1-Blue (23). Recombinant phages carrying 3Y1-specific cDNAs were identified by plaque hybridization (23). The phage library was plated, transferred onto two sets of nylon membranes, and hybridized with one of two different random primer-labeled cDNAs derived from 3Y1 or v-*src*-3Y1 cells. Plaques showing preferential hybridization signals with the 3Y1 cDNAs were picked up and rescreened twice.

Northern Analysis. Total RNA (10 μg) was electrophoresed in 1% agarose/formaldehyde gels, transferred to nylon membrane filters, and immobilized by UV crosslinking. Hybridization probes were made from gel-purified DNA fragments that were labeled by random priming with the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP (23). After hybridization at 42°C for 20 hr in 6× standard saline citrate (SSC) containing 5× Denhardt's solution (25), 0.1% SDS, 50% formamide, and 100 μg of heat-denatured salmon sperm DNA per ml, the filters were washed sequentially in 2× SSC/0.1% SDS at room temperature for 10 min and in 0.1× SSC/0.1% SDS at 50°C for 20 min. Autoradiography was for 2–4 days at −70°C with an intensifying screen.

Preparation of Genomic DNA and Southern Hybridization. High molecular weight genomic DNA (23) was digested to completion with appropriate restriction enzymes and size-fractionated in 0.8% agarose gels. The DNA was transferred (26) to nylon membrane filters, which then were prehybridized for 6 hr at 65°C in 0.9 M NaCl/0.05 M phosphate, pH

Abbreviations: RSV, Rous sarcoma virus; SV40, simian virus 40.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. X66872).

7.4/5 mM EDTA/5× Denhardt's solution/0.2% SDS/10% dextran sulfate containing 200 μg of heat-denatured salmon sperm DNA per ml. Random hexamer-primed DNAs were radiolabeled and hybridized with membrane-bound DNA for 20 hr. The filters were rinsed in 1× SSC/0.2% SDS at 65°C for 20 min.

DNA Sequencing. Recombinant plasmids were isolated and used as templates for dideoxy sequencing (27) (United States Biochemical, Sequenase version 2.0). A nucleotide sequence homology search was performed with the EMBL data base.

***In Vitro* Transcription and Translation.** N03 cDNA was subcloned into the *Eco*RI site of the pTZ18R expression vector (Pharmacia). The plasmid was linearized with *Hin*dIII and 2 μg of it was used as the template for *in vitro* transcription with T7 RNA polymerase. The *in vitro* transcribed RNA was translated in a rabbit reticulocyte lysate (NEN) with [^{35}S]methionine. Protein products were analyzed by electrophoresis in an SDS/10–20% polyacrylamide gradient gel.

RESULTS

Isolation of Genes Specifically Expressed in 3Y1 Cells. We used a differential screening method to obtain genes exclusively expressed in normal rat fibroblast (3Y1) cells but not in 3Y1 cells transformed by RSV (v-*src*-3Y1). A λZAPII cDNA library was constructed from poly(A)$^+$ RNA prepared from the 3Y1 cells. Screening of ≈2.5 × 10^5 clones from the 3Y1 cDNA library yielded 6 clones that showed relatively strong hybridization signals with ^{32}P-labeled cDNA from the 3Y1 cells. The inserts of these clones were excised from the phage vectors into pBluescript plasmids and then used as probes to examine the expression of the corresponding genes in 3Y1 and v-*src*-3Y1 cells. Among the cDNA clones we obtained, we focused our attention on N03 cDNA. mRNA complementary to cDNA clone N03 (2.0 kb) was specifically detected in 3Y1 cells but not in v-*src*-3Y1 cells (Fig. 1). To investigate whether the observed down-regulation of N03 mRNA expression in v-*src*-transformed 3Y1 cells was simply due to rapid growth of transformed cells or due to transformation *per se* caused by v-*src*, we compared the amounts of N03 mRNA in growing and quiescent 3Y1 cells. The level of N03 mRNA was slightly lower in growing cells than in quiescent cells (Fig. 2). However, the decrease was far less than that observed in v-*src*-transformed cells. This result indicates that the negative regulation of N03 mRNA expression may be involved in v-*src*-induced cellular transformation.

Sequence Analysis of N03 cDNA. Because the cDNA insert (N03-2) was about 600 bp shorter than N03 mRNA, this cDNA insert was used to isolate additional cDNA clones

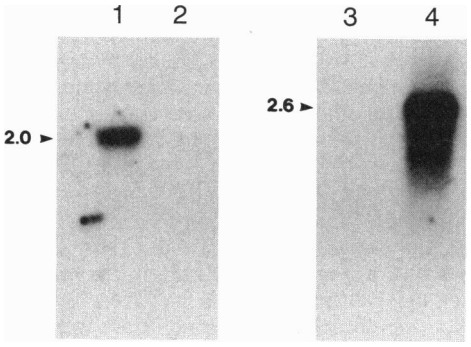


FIG. 1. Expression of the N03 gene in 3Y1 cells. Total RNA (10 μg per lane) from untransformed 3Y1 cells (lanes 1 and 3) or v-*src*-3Y1 cells (lanes 2 and 4) was electrophoresed in a 1% agarose/formaldehyde gel, transferred to nylon membrane, and probed with radiolabeled N03 cDNA (lanes 1 and 2) or v-*src* DNA (lanes 3 and 4).

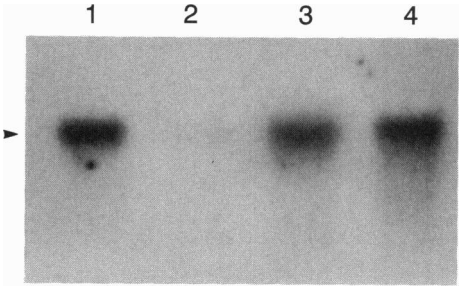
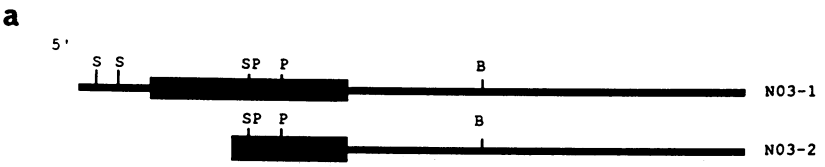


FIG. 2. Steady-state level of N03 mRNA accumulated in cells in different phases of growth. 3Y1 cells were cultured in DMEM containing 10% fetal bovine serum. Actively growing cells were harvested in early logarithmic phase. Quiescent cells were from confluent cultures kept for an additional 48 hr in DMEM containing 10% fetal bovine serum. Total RNA was isolated from the cells and Northern analysis was performed as in Fig. 1. Lanes: 1, 3Y1 cells used for cDNA library; 2, v-*src*-transformed 3Y1 cells; 3, actively growing 3Y1 cells; 4, quiescent 3Y1 cells.

from the 3Y1 cDNA library. Of the 11 positive clones analyzed, the insert sequence of the longest clone (N03-1) was about 400 bp longer than that of N03-2 (Fig. 3*a*). As the size of this cDNA clone was nearly consistent with the estimate of N03 mRNA obtained by Northern blot analysis, we tentatively concluded that it was close to full length and determined its complete nucleotide sequence (1788 bp; Fig. 3*b*). No sequence difference was noted between overlapping regions of N03-1 and N03-2. A putative polyadenylylation signal (AATAAA; ref. 28) is followed by a poly(A)$^+$ tract. In a homology search at the nucleotide sequence level, no significant similarity was detected between N03 cDNA and the genes compiled in the data base (EMBL, Release 25.0). An open reading frame extends from nucleotide 1 to a stop codon at position 535. This ATG is most likely the translation start site, as the sequence flanking this conforms to the consensus sequence for translation initiation proposed by Kozak (29, 30). This open reading frame (178 amino acids) has the capacity to encode a protein of 19,190 Da. When N03 cDNA was transcribed under the control of the T7 promoter and the resulting RNA was translated in the presence of [^{35}S]methionine in a rabbit reticulocyte lysate translation system, SDS/PAGE indicated that the molecular mass of the protein synthesized from N03 cDNA was ≈23 kDa (Fig. 4). This value was slightly larger than that predicted from the amino acid sequence of the open reading frame, most likely due to a proline-rich sequence (see below) affecting the electrophoretic mobility (31, 32). Thus, we concluded that this open reading frame does encode the authentic protein product. Although a homology search revealed that this amino acid sequence shares no structural similarity with those filed in the data base (Swiss-Prot, Release 15.0), the deduced polypeptide has several interesting features. It contains two putative casein kinase II target consensus sequences (Ser-Xaa-Xaa-Glu; ref. 33) at residues 46–49 and 89–92. In addition, there is one potential N-linked glycosylation site (Asn-Xaa-Thr; ref. 34) at residues 38–40. In the center of the peptide (residues 81–101), we found two characteristic DNA binding finger structures, repeated Cys-Xaa-Xaa-Cys or Cys-Xaa-Xaa-His sequence (35, 36), and a proline-rich sequence in the C-terminal region (31% proline, residues 141–169).

Expression of N03 mRNA in Other Types of Transformed Cells. As shown in Fig. 1, expression of N03 gene was down-regulated in v-*src*-3Y1 cells. We therefore analyzed N03 gene expression in a panel of various 3Y1 cell lines transformed by single oncogenes. Northern blot experiments showed (Fig. 5) that the N03 mRNA level in v-*mos*- and SV40-transformed cells was as low as the level observed in



b

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-194                                                        GCGCCTCGCCCCGC
-180  CCGGGGCCGCAGACGGCCAGCAGCGACAGACAGCCGAGCCTCGCGGGGCCGTCCCAGCCC
-120  GAGGACCCCGGGCCTGCGACCCCCGCGGCTCGCATCCCACTTTCTAAGACCTCTAAGAGG
 -60  ACGCGCCGGCCTCTTCGCGGCCACCGCGCGCGTTCCAGGGCTCCGGAGGTGACAGGCACA
   1  ATGCTTTGGGTCCTGGTGGGGACTGTTCTCCCTGTCATGTTGCTGGCTGCCCCACCACCC
   1  M  L  W  V  L  V  G  T  V  L  P  V  M  L  L  A  A  P  P  P
  61  ATCAACAAGCTGGCCCTGTTTCCGGACAAGAGTGCCTGGTGCGAAGCCAAGAACATCACG
  21  I  N  K  L  A  L  F  P  D  K  S  A  W  C  E  A  K  N  I  T
 121  CAGATTGTGGGCCACAGCGGCTGTGAGGCCAAGTCTATCCAGAACAGGGCGTGCCTAGGA
  41  Q  I  V  G  H  S  G  C  E  A  K  S  I  Q  N  R  A  C  L  G
 181  CAATGCTTCAGTTACAGCGTCCCCAACACCTTCCCGCAGTCCACAGAGTCCCTGGTGCAC
  61  Q  C  F  S  Y  S  V  P  N  T  F  P  Q  S  T  E  S  L  V  H
 241  TGTGACTCCTGCATGCCGGCCCAGTCCATGTGGGAGATTGTGACCTTGGAGTGCCCCGGC
  81  C  D  S  C  M  P  A  Q  S  M  W  E  I  V  T  L  E  C  P  G
 301  CACGAGGAGGTGCCCAGGGTGGACAAGCTGGTTGAGAAGATTGTACACTGCAGCTGCCAA
 101  H  E  E  V  P  R  V  D  K  L  V  E  K  I  V  H  C  S  C  Q
 361  GCCTGCGGCAAGGAACCCAGTCATGAGGGCCTGAACGTCTACATGCAGGGTGAAGATGGG
 121  A  C  G  K  E  P  S  H  E  G  L  N  V  Y  M  Q  G  E  D  G
 421  CCAGGCTCCCAGCCAGGATCGCACTCCCACTCCCATCCCCACCCTGGGTGCCAGACCCCT
 141  P  G  S  Q  P  G  S  H  S  H  S  H  P  H  P  G  C  Q  T  P
 481  GAACCTGAAGAACCTCCTGGAGCCCCTCAAGTTGAGGAAGAGGGGGCTGAGGACTGAGGG
 161  E  P  E  E  P  P  G  A  P  Q  V  E  E  E  G  A  E  D  *
 541  CCCCCAACTCTTCCTCCCCTCTTGTCCCCTGTGGAATGTCTGGTCCCACCCTCCAGGGAA
 601  GAGGGAGGGGAGAGGCAGAGGCCCCCCTCCCCCTCTGGCACTAGATGGACTTGGAATGTT
 661  GCCTGGTTGCCATGGAGATCTGAAGGGAGGGGCTGGAGCCAAGCTGCACGATTTAATATA
 721  TGCAAGATGAGGGGAGGAAGCAGACATCTTTGGGACTTTTCTTTTGGAAGGTGAAGTGTG
 781  GTCTTTCCTTTCAGCCTTCTGGAGAGGAGCCCTCTGGATAGAGAGGAAGCAGGTTGAGCT
 841  GCTGAGGATGACTGAGATCCTCAGATAGGGCAGGCCCAGGTCAGGACCCAGACAACGGAT
 901  GGGGCAGGATCCCCAGTGACGGGCAGAGGGTGGGTATCATGTCAGCATCCCCTTTGCAGG
 961  AGGATCTAGGTAGGAAGACAGCTGGCAGAAGTCGGAAACTCCAGCCCCATAGGTTTCCCT
1021  AAAGGCCTCCCCTCATGTAACCCCTTCAATGGAGTGGCTTAGGAGTCAGGCTGGGGCAGG
1081  ACCCTGCTGACTCAGGTGCCAGAGTGTCACTGAAGAAGAAAGAAGATCCCTCTCCCCCAC
1141  ACCCTGTCCCTCCCTCCAGAGATCTTAGCTCTTTGCTCTCCCCAAGTTCTGGACTCTTCT
1201  GAAAACAGCTGCAACCGGTCCTGCCGTCCTTGGGGCTGGTCTTTCTGAAAGAGGTGACAG
1261  TTTATCCCCATCACCTGGGACCGTAGTGGACAGGAGGCCCCTGCCTAGGGCCCCTGCTGA
1321  CCTCAGCTTTCTCTAGCTCCTTATTCTATCCACTCCTTCTTCCCAGCTGCACTTTAACCT
1381  TAGGGCAGGGCAGGTGGCGAGGAAGGGCCCTCTGTCTCCTTATCTGCTGGCACCATTGTG
1441  TCCAGGGGGTTGCCAGCTCACCCCCTCCCTGGGGGTGCCCCAGCCCATGGCTGTTTGACG
1501  AGAGCCTTAGAGCTTGTAACTAGTAGCCATCTCCCCTGAACCTGAGTGTTTCCATGAATA
1561  AAATGCCTTCAACGCCTCTACAAAAAAAAAAAAA
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FIG. 3. (*a*) Restriction map of N03 cDNA. Thin line shows 5′ or 3′ noncoding region; bold line indicates coding region. B, BamHI; P, *Pst* I; S, *Sma* I; SP, *Sph* I. (*b*) Nucleotide and amino acid sequence. The nucleotides and amino acids are numbered from the beginning of translation initiation. Stop codon is shown by asterisk. Potential casein kinase II target sequences (▼) and a putative N-linked glycosylation site (●) are indicated. Boxed region indicates the zinc-finger-like domain. Polyadenylylation signal is underlined.

v-*src*-transformed cells, although a substantial level of N03 mRNA was detected in v-Ha-*ras*-transformed cells. These observations suggest that negative regulation of N03 gene expression is not specific to v-*src*-induced transformation.

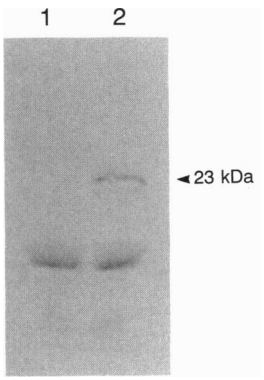

FIG. 4. *In vitro* transcription and translation of N03 cDNA. Cloning vector alone (pTZ18R, lane 1) or containing N03 cDNA (lane 2) was transcribed *in vitro* and translated in the rabbit reticulocyte lysate. Synthesized protein products were separated by SDS/10–20% PAGE.

Tissue Distribution and Expression. A Northern blot of total RNA from adult rat tissues was hybridized with the radiolabeled N03 cDNA (Fig. 6). Lung showed the highest expression; kidney, brain, and intestine showed moderate expression; testis and spleen exhibited N03 mRNA only upon longer exposure of the Northern blot; and expression was not detected in liver.

Southern Blot Analysis. To clarify the genomic organization of the N03 gene, genomic DNA from 3Y1 cells was digested with various restriction enzymes and hybridized with N03 cDNA as a probe (Fig. 7). The hybridization pattern was quite simple, which indicates that the gene is present as a single copy in the rat genome. In addition, the pattern of

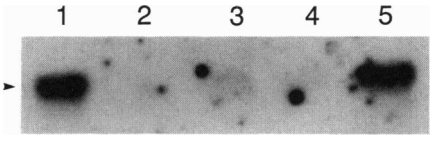

FIG. 5. N03 gene expression is also repressed in other types of transformed cells. Total RNA (10 μg per lane) derived from 3Y1 (lane 1), v-*src*-3Y1 (lane 2), v-*mos*-3Y1 (lane 3), SV40-3Y1 (lane 4), and v-Ha-*ras*-3Y1 cells (lane 5) was blotted on nylon membrane and hybridized with radiolabeled N03 cDNA.

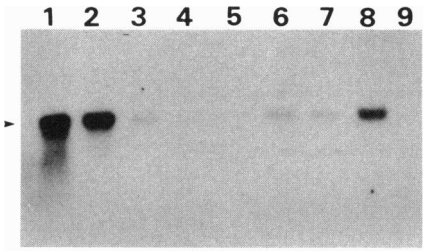

FIG. 6. Expression pattern of N03 mRNA. Northern blot of total RNA was hybridized with N03 cDNA. Autoradiogram was exposed for 3 days. Lanes: 1, NRK (cultured normal rat kidney cells); 2, 3Y1 cells; 3, intestine; 4, testis; 5, spleen; 6, brain; 7, kidney; 8, lung; 9, liver.

Southern blot hybridization for 3Y1 cells was identical to that of v-*src*-transformed 3Y1 cells. To investigate whether N03 gene sequence is structurally conserved among species, a Southern blot analysis was carried out on chicken and human genomic DNA treated with *Pst* I (Fig. 8). In each genomic DNA, definite and simple hybridization signals were detected; however, the intensity of bands derived from chicken and human DNA was very low. This observation implies that structurally related genes are present in these organisms also as a single-copy gene.

DISCUSSION

Identification of transformation-associated changes in cellular gene expression is an important step to elucidate the mechanisms underlying transformation. We used a differential screening procedure to obtain several cDNA clones whose expression was substantially down-regulated in v-*src*-transformed 3Y1 cells, and we have characterized one such clone, N03. N03 cDNA encodes a polypeptide of 178 amino acid residues whose primary structure shares no similarities with those filed in the data base.

The deduced N03 gene product contains two interesting structural features, the putative zinc-finger domain in the central part of the peptide and the proline-rich sequence in the C-terminal region. There is evidence that a proline-rich sequence can confer transcriptional activation (37). The zinc-finger proteins can be classified into several groups according to the number and position of the cysteine and histidine residues available for zinc coordination (35, 36). One of these groups has the form of Cys-Xaa$_2$-Cys-Xaa$_{13}$-Cys-Xaa$_2$-Cys and thus is referred to as the C4 family (36). Because the potential finger domain of the N03 gene product would be predicted to form a loop of 13 amino acids, it might be

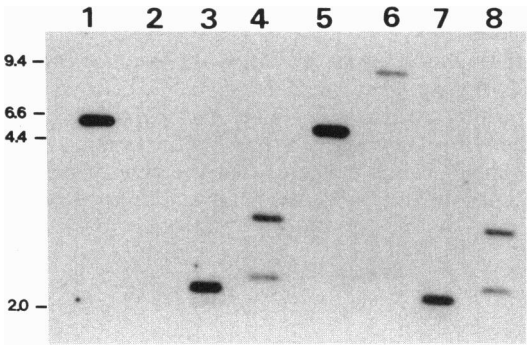

FIG. 7. Genomic Southern analysis. Genomic DNA from 3Y1 (lanes 1–4) and v-*src*-3Y1 cells (lanes 5–8) was completely digested with *Eco*RI (lanes 1 and 5), *Hin*dIII (lanes 2 and 6), *Pst* I (lanes 3 and 7), or *Bam*HI (lanes 4 and 8), electrophoresed in 0.8% agarose gel, transferred onto nylon membrane, and hybridized with radiolabeled N03 cDNA. Sizes are shown in kilobases.

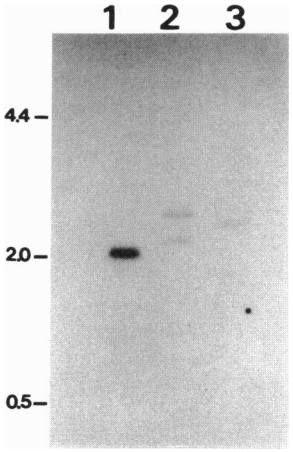

FIG. 8. Genes closely related to N03 are present in chicken and human genomes. Genomic DNA (10 μg per lane) was treated with *Pst* I and analyzed as described in Fig. 7. Lanes: 1, 3Y1 cells; 2, chicken embryonic fibroblasts; 3, human placental DNA. Sizes are shown in kilobases.

included in the C4 family with the exception that one of the cysteine residues has been replaced by histidine (38). We found no sequence homology between the putative finger domain of the N03 protein and the members of the C4 family (36, 39); however, in the finger domain of N03 and the Mel-18 gene product which has been reported to be a zinc-finger protein (40), 11 out of 27 amino acid residues are identical (Fig. 9). Our preliminary experiments revealed that N03 protein synthesized *in vitro* was able to bind to native salmon sperm DNA (data not shown), but it will be necessary to determine whether DNA binding takes place in a sequence-specific manner.

A genomic Southern experiment revealed that a structurally related gene exists in other species, including chicken and human. Therefore, we assume that the N03 gene product bears a universal function(s) essential to higher eukaryotes. In this regard, it will be interesting to examine whether down-regulation of N03 or its related gene also takes place in transformed cells derived from the other species.

The restriction pattern of the N03 gene in 3Y1 cells was quite the same as that in v-*src*-transformed cells (Fig. 7). This result suggests that the decreased level of N03 mRNA in v-*src*-3Y1 cells was not due to gross rearrangements and/or deletions occurring in N03 genomic DNA, although some minor changes, such as point mutation(s) in the regulatory regions, could not be ruled out. The v-*src* gene product (pp60$^{v\text{-}src}$) has been described as inducing the repression of several genes, including fibronectin, collagen, and tropomyosin genes (18–20). In addition, the expression of these genes is regulated at the transcriptional level (18–20, 41, 42). We do not know whether the level of N03 mRNA is reduced by a decrease in the rate of synthesis or an increase in the rate of breakdown. It will be necessary to perform nuclear run-on assays to clarify the regulatory mechanism of N03 gene expression.

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Mel-18  (10)TE-LNPHLMCALCGGYFIDATTIV--ECL-H(36)
            •• •  •  •  •          ••  ••  •
N03     (75)TESL-VH--CDSCMPAQSMW-EIVTLECPGH(101)
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FIG. 9. Sequence comparison in the finger domain between N03 protein and Mel-18 gene product (40). The sequences are aligned to obtain maximum homology. Matched amino acid residues are indicated by filled circles, and sites of core structure of cysteine and histidine residues are underlined. Numbers in parentheses represent positions relative to the amino-terminal end of the N03 or Mel-18 gene product.

We observed that the steady-state level of N03 mRNA was also decreased in v-*mos*- and SV40-transformed 3Y1 cells. The function and the intracellular distribution of the transforming proteins encoded by v-*mos* and SV40 are completely different from those of the protein encoded by v-*src*. The Mos protein resides in the cytosol and has protein-serine/threonine kinase activity (1, 43, 44). Sagata *et al.* (45, 46) have reported that c-Mos, the cellular counterpart of the v-Mos protein, might activate the maturation-promoting factor. In the case of SV40-transformed cells, the bulk of the transforming protein, the large tumor (T) antigen, is located in the nuclei (47). It has been supposed that this protein might be involved in DNA synthesis (48). In addition, a small fraction of the T antigen can associate with p53 and the retinoblastoma gene product, leading to the inactivation of their functions, so that the cells enter the growing state (49). These observations strongly suggest that three transforming proteins induce cellular transformation mediated by different signal transduction pathways. It is provocative that N03 gene expression is commonly suppressed in cultured rat cells transformed with completely different agents. If the function of the N03 gene product is necessary to prevent cells from entering the final stage of the transformation process, its absence could facilitate transformation.

The expression of the N03 gene was not down-regulated in v-Ha-*ras*-transformed 3Y1 cells. There is growing evidence suggesting that transformation by *src* depends on Ras protein (2, 50). If this were true in the present system, the amount of N03 mRNA should have also been decreased in v-Ha-*ras*-transformed 3Y1 cells, but this was not the case. This observation implies that the cellular transformation process caused by v-*src* might include at least two distinct pathways with respect to dependency on Ras protein.

The N03 gene was expressed in all normal rat tissues tested except liver. The levels of expression varied significantly among the different tissues. It is possible that N03 protein plays some important roles in the maintenance of the normal phenotype of each tissue in a tissue-specific manner. It has been postulated that the genes down-regulated in cancer cells include candidate tumor-suppressor genes (22). Therefore, it will be of great interest to investigate whether overexpression of the N03 gene in transformed cells can revert the transformed phenotype to a normal one.

We are grateful to Dr. M. Tagawa for valuable discussions. We thank Mrs. P. T. Froes for critical reading of the manuscript. This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture of Japan; by a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control, Japan; and by a grant from the Hamaguchi Foundation for the Advancement of Biochemistry.

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