The Human *cot* Proto-oncogene Encodes Two Protein Serine/Threonine Kinases with Different Transforming Activities by Alternative Initiation of Translation*

Masahiro Aoki\(\dagger\) , Fumihiko Hamada, Toshiro Sugimoto\(\ddagger\) , Shuji Sumida, Tetsu Akiyama\(\ddagger\) , and Kumao Toyoshima

From the Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, 3–1, Yamada-oka, Suita, Osaka 565, Japan

---

The *cot* gene is an oncogene encoding serine/threonine kinases isolated by DNA transfection assay. In this study, we isolated cDNA for the human *cot* proto-oncogene (proto-*cot* gene) and examined the structure and function of its gene products. The proto-*cot* gene has an open reading frame encoding 467 amino acids of which the first 397 amino acids are identical to those in the corresponding part of the *cot* gene. The protein products of the proto-*cot* gene were identified as 58- and 52-kDa proteins with intrinsic protein serine/threonine kinase activity. These two protein species were suggested to be generated by alternative initiation from two AUGs. The 58- and 52-kDa proteins are both localized predominantly in the cytosol, but the 58-kDa protein has a shorter half-life than the 52-kDa protein, suggesting the importance of the amino-terminal domain in regulating the stability of the proto-**cot** protein. More interestingly, the 58-kDa protein showed stronger transforming activity than the 52-kDa protein, although this activity was much weaker than that of the *cot* oncprotein. Thus, the amino-terminal domain of the Cot protein may be necessary for cellular transformation, whereas the carboxyl-terminal domain may negatively regulate the transforming activity.

The cascade of phosphorylation events by protein kinases is considered to play important roles in signal transduction from the membrane to the nucleus and/or in cell cycle control (1–6). Tyrosine phosphorylation is an early event in the cascade (7); protein tyrosine kinase activity associated with cell surface growth factor receptors is essential for their role in initiating cell proliferation and differentiation (8–10). Constitutive activation of tyrosine kinases by structural aberrations are well known to result in cell transformation.

Several serine/threonine kinases have been found to function downstream of the tyrosine kinases. The initial tyrosyl phosphorylation events are followed by activation of several serine/threonine kinases including protein kinase C (11), Raf-1 kinase (12–14), mitogen-activated protein kinase (15–18), and ribosome S6 kinase (15, 19, 20). Thus, constitutive activation of the serine/threonine kinases by structural alteration is also expected to result in cell transformation. Indeed, Raf-1 kinase, Mos kinase, and Akt kinase were identified as products of the viral oncogenes (21–25). Transfection assays with NIH3T3 cells showed that Raf-1 kinase and B-Raf kinase could be activated by truncation of the amino-terminal region (26–29).

The *cot* gene encoding serine kinase was first isolated as a transforming gene by DNA transfection with the genomic DNA of TCO-4 cells, a human thyroid cancer cell line, on to a hamster cell line SHOK (30, 31). The *cot* gene has an open reading frame coding for 417 amino acids (31). The protein products of the *cot* gene were identified as 52- and 46-kDa cytoplasmic serine kinases differing in their amino-terminal structures (32). Analysis of a genomic clone obtained from a human placenta genomic DNA library indicated that the *cot* gene had suffered a gene rearrangement within the last coding exon of the proto-**cot** gene, probably during the initial transfection experiment (31).

In this study, we isolated cDNA for the human proto-**cot** gene and examined the structure and function of its gene products.

**EXPERIMENTAL PROCEDURES**

**Cell Lines—SHOK** cells and their transfectants were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Hyclone). Human cell lines used for Northern blot were: HEP, MIZ-4, and MIZ-5 (embryonal fibroblast); TCO-1 and HATO (thyroid carcinoma); HT29 (colon carcinoma); HOS (osteosarcoma); Hela (cervical carcinoma); MCF-7 (mammary carcinoma); HaT 12 (embryonal fibroblast transformed in *vivo*); EJ (bladder carcinoma); U937 (histiocytic lymphoma); A431 (vulval carcinoma); PLC/PRF/5 (hepatoma); SENTO (thyroid carcinoma); K562 (chronic myelogenous leukemia).

**DNA and RNA Analysis—** Total cellular RNA was isolated by the acid-guanidium thiocyanate-phenol-chloroform extraction method (33). Poly(A)* RNA was obtained by using Oligotex DT30 (Takara). RNA samples (20 μg of total RNA or 5 μg of poly(A)* RNA) were electrophoretically separated on a 1% formaldehyde-agarose gel and transferred to nylon membrane filters (Hibond-N, Amerham). Filters were hybridized with 32P-random prime-labeled fragment of the cot oncogene for 14 h at 42 °C in buffer containing 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml of salmon sperm DNA (28). The filters were washed twice in 2 × SSPE and once in 0.2 × SSPE.
FIG. 1. Structure of the proto-cot cDNA. A, nucleotide and deduced amino acid sequences of the proto-cot cDNA. The open reading frame of the proto-cot cDNA encodes a 467-amino acid polypeptide. The initiator ATG codons for the 58- and 52-kDa proteins are shown in bold-faced letters with an underline. The termination codons are marked by asterisks and bold-face letters. The sequence specific for the proto-
at 42 °C and twice in 0.2 × SSPE at 60 °C and then exposed to Kodak X-Omat XAR film at -80 °C for 7 days. High molecular weight DNA was prepared by the phenol-chloroform method. DNA samples (10 μg) were digested with EcoRI, electrophoretically separated on a 0.7% agarose gel, and then transferred to nylon filter. The filter was washed extensively and then exposed to Kodak X-Omat XAR film at -80 °C for 3 days.

Isolation and Nucleotide Sequence Determination of cDNA for the cot Proto-oncogene—A PL/C/PRF5 library in λ-ZAP was constructed from 5 μg of poly(A)⁺ RNA according to the instructions of the manufacturer (Stratagene). The library (4 × 10⁶ phages) was screened with ³²P-random prime-labeled cot probes corresponding to nucleotides 389-748. Twelve positive plaques were purified, and the cDNA inserts were isolated as plasmids by superinfection with A46 peptide (4 μg). C, two-dimensional phosphoamino acid analysis of Cot proteins phosphorylated in vivo. The bands of 58- and 52-kDa phosphoprotein in panel B were processed for two-dimensional phosphoamino acid analysis as described under "Experimental Procedures." The positions of ninhydrin staining of marker phosphoamino acids are indicated by dotted circles.

FIG. 2. Identification of metabolically labeled Cot protein with anti-peptide antibodies. SHOK cells and SHOK cells transfected with the proto-cot gene (SD14A) were labeled for 2 h with ³⁵S]methionine (A) or for 3 h with ³²P]orthophosphate (B) and then lysed in solubilization buffer. The Cot proteins were immunoprecipitated with 1 μg of anti-A46 and analyzed by 10% SDS-PAGE followed by fluorography (A) or autoradiography (B). Pep(+) antibodies were blocked by preincubation with A46 peptide (4 μg). M8 used to raise antibodies are also shown.

Cot gene is boxed. B, predicted structures of the proto-cot gene product and cot gene product. The catalytic domains are marked by shadowed boxes and the Cot oncoprotein-specific region starting from the rearranged point is shown by a black box. The ATG initiation codons (M), and the conserved sequence motifs of the protein kinases (37) are also indicated. The locations of the synthetic peptides A46, A48, A49, and M8 used to raise antibodies are also shown.
FIG. 3. Protein kinase activity of the Cot proteins. A, immune complex kinase assay of the Cot proteins with anti-A46. Lysates were prepared from SHOK cells, SHOK cells transfected with the cot proto-oncogene (SDI4A), and tertiary transfecants (SHOK/Ter). The cot proteins were immunoprecipitated from cell lysates with anti-A46, subjected to protein kinase assay, and analyzed by SDS-PAGE followed by autoradiography. Pep(+), antibodies were blocked by preincubation with A46 peptide. B, phosphoamino acid analyses of the autophosphorylated proto-Cot proteins. 32P-Labeled bands of the Cot proteins in panel A were excised from the gel, acid hydrolyzed in 6 N HCl, and processed for one-dimensional phosphoamino acid analysis as described under “Experimental Procedures.” C, immune complex kinase assay of the Cot proteins immunoprecipitated from SF9 cells infected with recombinant baculovirus vector harboring the proto-cot cDNA (lanes 3, 4, 7, and 8) or control SF9 cells (lanes 1, 2, 5, and 6). Immunoprecipitation was performed with the anti-A46 (lanes 1-4) or anti-A49 (lanes 5-8). In lanes 2, 4, 6, and 8, the antibodies were blocked by preincubation with the peptides used for immunization. D, immunoprecipitation of the Cot protein from 35S-labeled SF9 cell extracts. Lysates and antibodies used were the same as those used in C.
Colonies were then selected with G418 at 500 μg/ml. For other cDNA clones except for clone ME, the 5’ ends were deleted to the BspHI site, and then the fragment containing the coding region was subcloned into A444 with pBamHI linker.

Focus Forming Assays—cDNA clones subcloned into A444 vector (1 μg of DNA) were transfected into SHOK cells by use of Transfectum (BIF Biotechnics, France). Transfectants were maintained in Dulbecco’s modified Eagle’s medium with 3% fetal calf serum, and foci were counted 14 days after cells reached confluence.

RESULTS

Expression of the cot Gene in Human Cell Lines—To isolate cDNA for the proto-cot gene, we first examined the expression of this gene in various human cell lines by Northern blot analysis using the cot cDNA as a probe. Relatively high expression of 2.7-kilobase cot mRNA was detected in a hepatocellular carcinoma cell line PLC/PRF5. Weak signals were detectable in the spleen and other cell lines on analyses of samples of 5 μg of poly(A)+ RNAs (data not shown).

After digestion of genomic DNA of PLC/PRF5 with EcoRI and hybridization of the products with full-length cot cDNA probe, three bands of 2.4, 6.7, and 7.5 kilobases were obtained (data not shown). These bands are identical to those previously obtained from human placenta, suggesting that no gross gene rearrangement had occurred in these cell lines.

Isolation of Proto-cot cDNA—A cDNA library prepared from PLC/PRF5 RNA (4 × 10⁶ A-ZAP recombinant phages) yielded 12 clones that gave strong hybridization signals with cot cDNA probes. We determined the nucleotide sequence of the longest cDNA clone (RF) carrying a 2.7-kilobase insert (Fig. 1A). A poly(A) stretch preceded by two polyadenylation signals, AATAAA (position 2330-2335 and 2376-2381), suggested that this clone carries the complete 3′ end. The proto-cot cDNA has a long 5′ non-coding region with many AUG codons followed by in-frame terminator codons. The longest open reading frame from nucleotides 1 to nucleotide 1401 is capable of coding for a serine/threonine kinase of 467 amino acid residues. The nucleotide sequence from the initiator ATG to nucleotide 1191 in the 3′ portion of the coding region is identical to the corresponding part of the cot gene. However, the sequence from nucleotide 1192 to the poly(A) stretch is completely different from the 3′ part of the cot gene.

The predicted structures of the proto-cot gene and cot gene are shown in Fig. 1B. Regions corresponding to the synthetic peptides used to generate antibodies are also indicated in this figure.

The Proto-cot Gene Encodes 58- and 52-kDa Phosphoproteins—For identification of proto-cot gene products, SHOK cells were transfected with the proto-cot cDNA subcloned into a mammalian expression vector with the human β-actin promoter. The cell line SD14A expressing the proto-cot mRNA was metabolically labeled with [35S]methionine or [32P]orthophosphate, lysed, and then subjected to immunoprecipitation with antibodies against the synthetic peptide A46 (anti-A46) (32). Anti-A46 precipitated two proteins of 58 and 52 kDa from both [35S]-labeled and [32P]-labeled cell lysates (Fig. 2, A and B). Precipitation of these proteins was completely blocked by preincubation of the antibodies with peptide A46, and these bands were not detectable in lysates of the parental SHOK cells. Phosphoamino acid analysis revealed that both proteins were phosphorylated mainly on serine residues (Fig. 2C).

The Proto-cot Gene Products Are Serine/Threonine Kinases—Incubation of the immunoprecipitates prepared from SD14A cell extracts with [γ-32P]ATP resulted in the phosphorylations of 58-, 52- and 110-kDa proteins, whereas no bands were obtained from the parental SHOK cells (Fig. 3A). On the other hand, phosphorylation of the 52- and 46-

![Figure 4](image-url)  
**Fig. 4.** Both 58- and 52-kDa proto-Cot proteins are expressed in K562 and HL60 cells. A, immune complex kinase assay of the Cot proteins with anti-A46. Lysates were prepared from SD14A (2 × 10⁶ cells), HL60 (5 × 10⁶ cells), and K562 (5 × 10⁷ cells). The proto-Cot proteins were immunoprecipitated from these cell lysates with anti-A46, subjected to protein kinase assay, and analyzed by SDS-PAGE followed by autoradiography. Pep (+), antibodies were of the Cot proteins with anti-A46. Lysates were prepared from SD14A and separated on a 16% SDS-polyacrylamide gel. The amounts of V8 proteases used were 0.01 mg (lanes 1, 3, 5, and 7) and 0.2 mg (lanes 2, 4, 6, and 8), respectively. was fixed with 50% methanol and then washed with distilled water. The washed gel was equilibrated with buffer A (125 mM Tris-HCl, pH 6.8, 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.1% SDS, and 10% glycerol) and examined by autoradiography. The bands of Cot proteins were excised from gels and homogenized in 100 μl of buffer A. Samples of 40 μl of the homogenates were put into the slots of 16% polyacrylamide gel and proteins were digested with Staphylococcus aureus V8 protease (0.01 or 0.2 mg) and separated on a 16% SDS-polyacrylamide gel. The amounts of V8 proteases used were 0.01 mg (lanes 1, 3, 5, and 7) and 0.2 mg (lanes 2, 4, 6, and 8), respectively.
FIG. 5. Alternative initiation of translation of the proto-Cot protein. A, immune complex kinase assay of the Cot proteins immunoprecipitated with three different antibodies. The Cot proteins were precipitated from lysate prepared from SD14A cells with three region-specific anti-peptide antibodies, subjected to protein kinase assay, and then analyzed by SDS-PAGE followed by autoradiography. The locations of the synthetic peptides A46, A47, and M8 used to raise antibodies are shown in Fig. 2B. B, immunoprecipitation of the Cot proteins synthesized in vitro. A plasmid vector containing proto-cot cDNA downstream of the T7 promoter was transcribed and translated in vitro in the presence of [%]methionine as described under “Experimental Procedures.” The labeled Cot proteins were then immunoprecipitated with anti-A46 or anti-A48 in the presence (+) or absence (−) of the peptides used for immunizations. The immunoprecipitates were analyzed by 10% SDS-PAGE followed by fluorography. C, immunoprecipitation of the mutant Cot proteins synthesized in vitro. The wild type (lane 1) and mutated cot cDNA constructs (lane 2, mutant MC with a mutation at the third AUG codon; lane 3, mutant MD with a mutation at the fourth AUG codon; lane 4, mutant ME with a deletion of the first and second AUG codons) were subjected to transcription and translation in vitro under the control of T7 promoter. The labeled Cot proteins were immunoprecipitated with antibodies against A46 and analyzed by 10% SDS-PAGE followed by fluorography.

FIG. 6. Cytoplasmic localization of the Cot protein. SD14A cells were labeled with [%]methionine for 3 h and then separated into membrane, cytoplasmic, and nuclear fractions as described under “Experimental Procedures.” The Cot protein was immunoprecipitated with antibodies against A46 in the presence (+) or absence (−) of A46 peptide. The immunoprecipitates were analyzed by 10% SDS-PAGE.

FIG. 7. Stability of the Cot polypeptides. SD14A cells were pulse-labeled with [%]methionine for 30 min and chased for appropriate times in the presence of an excess of nonradioactive methionine (0.5 mM). The Cot proteins were immunoprecipitated with anti-A46 and subjected to 10% SDS-PAGE. kDa proteins were observed in immunoprecipitates from SHOK/Ter cells transformed by the cot gene, as described previously (32). Phosphoamino acid analysis showed that the 58-kDa protein was phosphorylated mainly at serine residues, whereas the 52-kDa protein was phosphorylated at both serine

Human cot Proto-oncogene
and threonine residues (Fig. 3B). Phosphorylations of the 58- and 52-kDa proteins were also detected using the proto-Cot protein immunoprecipitated from SF9 cells infected with recombinant baculovirus vector containing the proto-cot cDNA (Fig. 3C, lane 3). The Cot protein also phosphorylated an exogenous substrate protein, histone H2B, in vitro (data not shown).

To confirm that this kinase activity is really intrinsic to the Cot protein, we prepared antibodies against synthetic peptide A49 (155-165) corresponding to the putative ATP binding region (36). The antibodies (anti-A49) could immunoprecipitate the comparable amounts of the Cot protein (Fig. 3D, lane 7) as precipitated with anti-A46 (lane 3). However, phosphorylation of the 58- and 52-kDa proteins were not detected with the Cot protein precipitated with anti-A49 (Fig. 3C, lane 7), indicating that the kinase activity of the Cot protein was inhibited by these antibodies. Thus, phosphorylations of the 58- and 52-kDa proteins detected in the above experiments are considered to be due to the intrinsic kinase activity of the Cot protein.

Both 58- and 52-kDa Proto-Cot Proteins Are Expressed in K562 and HL60 Cells—We next examined whether both 58- and 52-kDa proto-Cot proteins are also present in cells normally expressing this gene. Although the level of expression of the proto-Cot protein was very low in the cell lines examined, immune complex kinase assay enabled us to detect both forms of proto-Cot proteins in human erythroleukemic K562 and promyelocytic HL60 cells. (Fig. 4A) The 58- and 52-kDa proteins were not detected with anti-A46 which had been preadsorbed with peptide A46. To further confirm that these proteins are the proto-Cot gene products, we performed peptide mapping analysis of these phosphoproteins. As shown in Fig. 4B, the patterns generated from the 58- and 52-kDa phosphoproteins detected in K562 cell lysate were quite similar to those generated from the proto-Cot proteins detected in SD14A cell lysate. These results indicate that the proto-Cot proteins are expressed as the 58- and 52-kDa proteins in cells normally expressing proto-cot gene.

The 58- and 52-kDa Proteins Are Generated by Alternative Initiation of Translation—To determine the structural differences between the 58- and 52-kDa proto-Cot proteins, we compared the reactivities of these proteins with two antipeptide antibodies directed against the amino- and carboxyl-terminal portions, respectively, of the Cot protein. In immune complex kinase assay, antibodies against the amino-terminal peptide A48 detected autophosphorylation of the 58-kDa protein but not the 52-kDa protein, while antibodies against the carboxyl-terminal peptide M8 precipitated both protein species like antibodies against peptide A46 that recognizes the middle portion of the Cot protein (Fig. 5A). Similar results were obtained with the proto-Cot proteins generated by in vitro transcription and translation of the proto-cot cDNA. Of the three major proteins of 58, 52, and 48 kDa precipitated with anti-A46, only the 58-kDa protein was precipitated with polyclonal antibodies against the amino-terminal peptide A46 (Fig. 5B). These results suggest that the 58- and 52-kDa proteins differ in their amino-terminal structures.

To determine whether the 52-kDa protein is produced by alternative initiation of translation, we generated several mutations at putative initiation sites. In mutants MC and MD, the third (Met-30) and fourth (Met-47) ATG codons, respectively, were replaced by the CAG codon (Gln) by site-directed mutagenesis. In mutant ME, the first and second ATG codons were removed by deletion of the 5' portion of the coding region down to the DraI site (nucleotides 60-65). These mutants were subjected to transcription and translation in vitro. As shown in Fig. 5C, mutant MC generated the 58- and 48-kDa proteins but not the 52-kDa protein, whereas mutant MD generated all three proteins, and mutant ME generated the 52- and 48-kDa proteins but not the 58-kDa protein. These data clearly indicate that synthesis of the 52-kDa protein is initiated from the third AUG codon, which corresponds to Met-30.

The Cot Proteins Are Localized in the Cytosol—For elucidation of the subcellular localization of the proto-Cot proteins, SD14A cells labeled with [35S]methionine were separated into crude membrane, cytosol, and nuclear fractions, and lysates of these fractions were subjected to immunoprecipitation with anti-A46 (Fig. 6). The 58- and 52-kDa proteins were both detected mainly in the cytosol fraction. In K562 and HL60 cells, both proteins were also detected in the cytosol fraction (data not shown).

The 52-kDa Protein Is More Stable than the 58-kDa Protein, But Both Have Short Lives—We next compared the stabilities of the two forms of proto-Cot proteins in vivo by pulse-chase experiments using SD14A cells. The cells were
Human cot Proto-oncogene

FIG. 9. Detection of the [\textsuperscript{35}S]methionine-labeled Cot proteins (A) and their autophosphorylation activities (B) in SHOK cells transformed with the wild type and mutated cot constructs. SHOK cells transfected with the vector without the insert (lanes 1 and 2), the normal proto-cot cDNA (lanes 3 and 4), the proto-cot cDNA with a mutation at the third ATG (lanes 5 and 6), deletion of the first and second ATGs (lanes 7 and 8) or the cot cDNA (lanes 9 and 10). Stable transformants were obtained from G418-resistant foci as described under “Experimental Procedures.” A, the Cot proteins were immunoprecipitated with anti-A46 from the established cell lines labeled with \([\textsuperscript{35}S]\)methionine and analyzed by 10% SDS-PAGE as described in the legend to Fig. 3. B, the Cot proteins immunoprecipitated with anti-A46 were subjected to immune complex kinase assay as in Fig. 3. In lanes 2, 4, 6, 8, and 10, anti-Cot antibodies preadsorbed with A46 peptide were used for precipitation.

TABLE I
Tumorigenicity of SHOK cells transformed by cot genes in nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gene products</th>
<th>Mice with tumors</th>
<th>Latency</th>
<th>weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>4G2</td>
<td>None</td>
<td>0/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF1</td>
<td>58 and 52</td>
<td>5/5</td>
<td>1–2</td>
<td></td>
</tr>
<tr>
<td>CF1</td>
<td>58</td>
<td>5/5</td>
<td>1–2</td>
<td></td>
</tr>
<tr>
<td>EF5</td>
<td>52</td>
<td>4/6</td>
<td>3–4</td>
<td></td>
</tr>
<tr>
<td>TF8</td>
<td>52 and 46</td>
<td>5/5</td>
<td>1–2</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Cell lines were established from SHOK cells transfected with the expression vector containing the wild type cot proto-oncogene (AF1), the cot proto-oncogene with a mutation at the 3rd ATG (CF1), the cot proto-oncogene with a deletion of the 1st and 2nd ATGs (EF5), the cot oncogene (TF8), and the vector only (4G2).

labeled with \([\textsuperscript{35}S]\)methionine for 30 min and then cultured in the absence of \([\textsuperscript{35}S]\)methionine. At the times indicated in Fig. 7, the cells were harvested and lysed and the lysates were subjected to immunoprecipitation with anti-A46. The 58- and 52-kDa proteins were found to have short half-lives of 10 and 30 min, respectively.

The Proto-cot Gene Has Low But Detectable Transforming Activity on SHOK Cells, and This Activity Is Mainly Due to the 58-kDa Protein—Finally, we examined the transforming activity of the proto-cot gene. Wild type or mutant proto-cot cDNA or cot cDNA under control of the \(\beta\)-actin promoter were transfected into SHOK cells, and transformed foci were counted (Fig. 8). The proto-cot cDNA reproducibly exhibited transforming activity (60 focus-forming units (ffu)/\(\mu\)g of DNA), although this activity was much lower than that of the cot cDNA (\(10^3\) ffu/\(\mu\)g of DNA). Vectors without a cDNA insert or with the mutant proto-cot cDNA encoding the Cot protein in which Lys-167, a putative ATP-binding site, was replaced by Arg showed no detectable transforming activity.

To determine the relative contributions of the two Cot proteins to the transforming activity, we examined the focus forming activities of mutant proto-cot cDNAs encoding only the 58-kDa (mutant MC) or 52-kDa (mutant ME) protein. Mutant MC induced as many transformed foci as the normal proto-cot gene (40 ffu/\(\mu\)g DNA). In contrast, mutant ME produced much fewer transformed foci (4 ffu/\(\mu\)g DNA) (Fig.)
We also established G418-resistant cell lines from SHOK cells transfected with the normal or mutant proto-cot cDNA or the cot cDNA. In these cell lines, we could detect the expression of the products of the transfected genes by immunoprecipitation experiment and immune complex kinase assay (Fig. 9). We inoculated these cells into nude mice to examine their tumorigenicities. As summarized in Table I, three cell lines expressing only 58-kDa protein as well as those expressing both the 58- and 52-kDa proteins induced tumors within 2 weeks in all mice tested when inoculated at 10^6 cells/mouse. Cell lines transformed with the cot oncogene also induced tumors within 2 weeks. However, the cell lines expressing only 52-kDa protein did not induce tumors within 2 weeks. These results suggest that both the amino-terminal domain and the carboxy-terminal domain are important in regulation of the activity of the cot gene.

**DISCUSSION**

In this study, we isolated cDNA of the human proto-cot gene and examined the structure and function of its gene products. Analyses of the cDNA and deduced amino acid sequences defined structural differences between the proto-cot protein and the Cot oncoprotein (Fig. 2); carboxy-terminal 70 amino acid residues of the proto-Cot protein were replaced by 18 totally different amino acid residues in the Cot oncoprotein. Activation of the cot oncogene was previously speculated to be due to DNA rearrangement that results in replacement of these carboxy-terminal residues (31). This speculation was clearly confirmed in this work by comparison of the transforming activities of the cot and proto-cot cDNAs (Fig. 8); the cot showed much higher transforming activity than the proto-cot. Although SHOK cells transformed by the cot gene produced a much lower level of Cot protein than those transformed by the proto-cot gene, their immunoprecipitates showed very high kinase activity (Fig. 9). Moreover, like other oncogenes with protein kinase activity, ablation of the kinase activity by mutation of the ATP-binding site caused loss of transforming activity (Fig. 8). These results suggest that constitutive expression of the kinase activity is essential for the transforming activity of the Cot oncoprotein and that this activity is potentiated by aberration of the carboxy-terminal domain. Thus the carboxy-terminal domain of the proto-Cot protein may cause negative regulation of its activity.

The products of the proto-cot gene were identified as 58- and 52-kDa proteins. The 52-kDa protein was concluded to be translated from the 3rd AUG codon at nucleotides 88–90, and this was confirmed by demonstrating suppression of synthesis of the 52-kDa protein by mutation of the third ATG (Met) to CAG (Gln) in an in vitro translation system with a rabbit reticulocyte lysate (Fig. 5C). The sequence around the first AUG codon, CAGTA ATG (nucleotides −5 to 4), which is preceded by the in-frame TGA codon at nucleotides −27 to −25, fits loosely to the Kozak’s consensus sequences C^/cC^ATG (38), in which ^C to position −3 and G at position +4 are very important. The sequence around the second AUG codon, AGTACATGA (nucleotides 1–9), does not fit this consensus sequence at all, whereas that around the third AUG codon, ACATTATG (nucleotides 83–91), basically conforms to the Kozak’s rule. It is, therefore, possible that some 40 S subunits stop and initiate at the first AUG codon, but some bypass the first and second sites resulting in initiation at the third AUG codon (38, 39).

Generation of the two protein species from different AUG codons in a single mRNA has been observed in several systems, especially animal virus systems (38, 40). In some cases, dual initiation is thought to generate two functionally distinct products from a single reading frame (41, 42). In other cases, upstream initiation is speculated to have some regulatory function without yielding a product that is itself functional (38). With respect to serine/threonine kinases, translation of p70^sek was reported to be initiated from different start sites with a nuclear transport signal located between them (20). The 58- and 52-kDa Cot proteins have different half-lives and transforming activities, suggesting the importance of their amino-terminal regions in regulation of their functions. In many cases, the amino-terminal region is known to be responsible for the subcellular localization of proteins, but the 58- and 52-kDa proteins were both found to be localized in the cytosol, like other serine/threonine kinases with transforming activity (43–45). Further study is needed on the functional relationship of these two proteins.

A pulse-chase experiment revealed that a half-life of the 58-kDa protein is much shorter than that of the 52-kDa protein. This may be why the 52-kDa protein is the major component in the steady state. The half-lives of intracellular proteins have been found to be related to their amino-terminal residues (45–47). Recently, Nishizawa et al. showed that the amino acid residues around the 3rd AUG of the cot RNA are sensitive to translation (30). This result is consistent with the fact that 58-kDa has a short half-life (48). However, both the 52- and 58-kDa proteins have the same amino acid, glutamic acid, at this position, indicating that other factors are involved in determining the stability of Cot proteins. In connection with the short life of the 58-kDa protein, it is interesting that we recently found that this 58-kDa kinase was activated specifically during the S and G2/M phases of the cell cycle, suggesting that its short half-life may be responsible for its rapid disappearance in cells entering the G1 phase.

Another interesting finding was that the 58-kDa protein is autophosphorylated at serine residues whereas the 52-kDa protein is autophosphorylated at both serine and threonine residues in vivo. Thus, the 29-aminoterminal amino acid residues in the 58-kDa protein may prevent autophosphorylation of the residues autophosphorylated in the 52-kDa protein. Further analysis of the sites and effects of autophosphorylation are required to determine the physical significance of phosphorylation of Cot proteins.

**Acknowledgments**—We thank R. Ohara, T. Ohuchi, and S. Kamada for helpful discussions and Y. Sakamoto and Y. Noda for preparation of the manuscript.

**REFERENCES**

16. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E.,