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A fusion protein formed by L-*myc* and a novel gene in SCLC

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Oncogenic activation of *myc* genes in human cancer involves deregulated expression of myc proteins with no major structural alterations. Here two independent small cell lung carcinoma (SCLC) cell lines were found to express similar novel proteins antigenically related to L-*myc*. cDNAs corresponding to these proteins were cloned and shown to encode chimeric polypeptides with amino-terminal sequences from a novel gene named *rlf* joined to the L-*myc* protein. Although the chimeric mRNAs were shown to be identical, they result from distinct DNA rearrangements. The L-*myc* fusion protein may represent another activation mechanism of the *myc* proto-oncogenes.

Key words: fusion protein/lung cancer/*myc* oncogenes

Introduction

Members of the *myc* gene family have been found to be activated in several human tumors due to DNA rearrangements, such as chromosomal translocations or gene amplification (Klein and Klein, 1985; Cory, 1986; Alitalo and Schwab, 1986; Saksela, 1990). DNA amplification of the L-*myc* gene is commonly detected in small cell lung cancer (SCLC) cell lines and primary tumors (Nau *et al.*, 1985; Johnson *et al.*, 1988; Takahashi *et al.*, 1989), and less frequently in other lung tumors (Shiraishi *et al.*, 1989). With the exception of most Burkitt's lymphomas, where synthesis of one of the two normal c-*myc* polypeptides is abrupted (Hann *et al.*, 1988), activation of the *myc* genes in human tumors has not been found to involve structural changes in the proteins encoded by the *myc* genes.

The *myc* genes encode nuclear phosphoproteins (Hann and Eisenman, 1984; Ramsay *et al.*, 1986; De Greve *et al.*, 1988; Saksela *et al.*, 1989; Mäkelä *et al.*, 1989), which contain conserved structural features, such as the basic helix–loop–helix (Murre *et al.*, 1989) and leucine zipper (Landschultz *et al.*, 1988) domains necessary for transforming activity (Stone *et al.*, 1987), for sequence-specific DNA binding (Blackwell *et al.*, 1990; Prendergast and Ziff, 1991), and for transcriptional autoregulation by the c-*myc* gene (Penn *et al.*, 1990). Such domains are also shared with several recently characterized transcription factors, suggesting similar functions for the myc proteins.

Here we describe molecular characterization of novel L-*myc* related proteins from SCLC. Our results show that these proteins are similar chimeric transcription units consisting of L-*myc* and a rearranged novel gene. The findings suggest that the chimeric proteins may be important in the pathogenesis of certain lung cancers.

Results

A novel L-myc related 72–77 kd polypeptide in GLC28 and CORL47 cells

Two different *myc*-specific antibodies (pan-*myc* and NCMII274) were used to characterize L-*myc* proteins from SCLC cell lines with L-*myc* gene amplifications. In contrast to the expected 62–67 kd L-*myc* protein triplet (Saksela *et al.*, 1989) seen in cell lines U1690 and CORL88, immunoprecipitation analyses of cell lines GLC28 and CORL47 revealed a larger 72–77 kd protein triplet (Figure 1A). Both the normal L-*myc* protein triplet and the 72–77 kd triplet could be converted to a single faster migrating polypeptide band by a phosphatase treatment, indicating that in both cases the polypeptide heterogeneity was due to phosphorylation (Figure 1B).

Cells with the novel protein express abnormal L-myc mRNAs

Northern blotting analysis of cell lines expressing the normal L-*myc* protein showed two major (3.8 and 3.6 kb) and two minor (2.4 and 2.2 kb) L-*myc* mRNAs, which result from differential splicing and alternative use of polyadenylation sites (Figure 2A; Kaye *et al.*, 1988). Interestingly, both cell

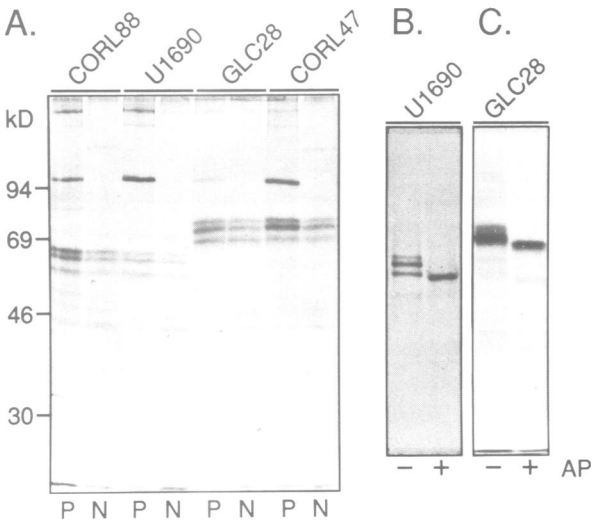

Fig. 1. Identification of novel 72–77 kd L-*myc* proteins from GLC28 and CORL47 cells. **A.** SDS–PAGE analysis of immunoprecipitates with pan-*myc* (lanes P) or NCM II 274 (lanes N) antibodies from the indicated cell lines. Immunoprecipitates from U1690 (**B.**) or GLC28 (**C.**) cells were treated with alkaline phosphatase (lanes +) or buffer (lanes –) prior to electrophoresis.

lines with the abnormal L-*myc* proteins expressed two transcripts of 3.7 and 3.2 kb (Figure 2B). The smaller transcripts did not contain L-*myc* second exon sequences, since they were only detected with the L-*myc* exon III probe. Lack of the normal short form transcripts (2.2 and 2.4 kb) from both cell lines is notable because of the distinct coding capacity of these mRNAs (Kaye *et al.*, 1988; Ikegaki *et al.*, 1989).

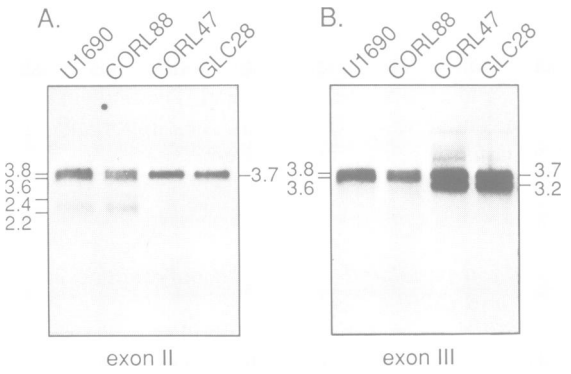

Fig. 2. Cell lines with the novel L-*myc* protein express aberrant L-*myc* mRNAs. **A.** 5 μg of polyadenylated RNA was subjected to denaturing gel electrophoresis, blotting, and hybridization with a random-primed L-*myc* second exon *Nar*I−*Bss*HII fragment. The normal pattern of four L-*myc* transcripts (3.8, 3.6, 2.4 and 2.2 kb) is detected in the U1690 and CORL88 cell lines, while the GLC28 and CORL47 cells express aberrant transcripts of 3.7 kb. **B.** As A, except that an L-*myc* third exon *Bam*HI−*Hin*cII probe was used. In U1690 and CORL88 cells only the 3.6 and 3.8 kb transcripts which contain third exon sequences are detected. In CORL47 and GLC28 cells an additional 3.2 kb transcript is seen.

Isolation of L-myc fusion cDNAs from GLC28

To characterize the abnormal transcripts further, a cDNA library was constructed from GLC28 mRNA in λgt10. Approximately 8×10^5 phage were screened, and 20 clones hybridizing to probes from either the second or third exon of L-*myc* were isolated. Partial sequence analysis revealed two distinct classes of cDNA clones. Neither of these contained sequences from L-*myc* exon I; instead both of them had identical novel sequences immediately 5′ of either exon II or exon III, as shown in Figure 3A and B. The structure of the clones strongly suggested that they represent the 3.2 and 3.7 kb mRNAs detected in Northern blotting analysis in both GLC28 and CORL47 cells. The novel sequence found in both types of clones represents an upstream exon, which is joined by alternative splicing to exon II or III. Thus transcription is initiated from the upstream exon, and because L-*myc* exon I does not have a splice acceptor site, it is not included in the mature mRNA. Initiation of transcription from the normal exon I of L-*myc* is undetectable. Although these results do not rule out the presence of more than one novel exon, the size of the mRNAs detected in Northern blotting suggests that the cDNA clones are almost full-length. The finding of several independent cDNA clones with almost identical 5′ ends also supports this conclusion (data not shown).

In addition to the upstream exon, both cell lines with the

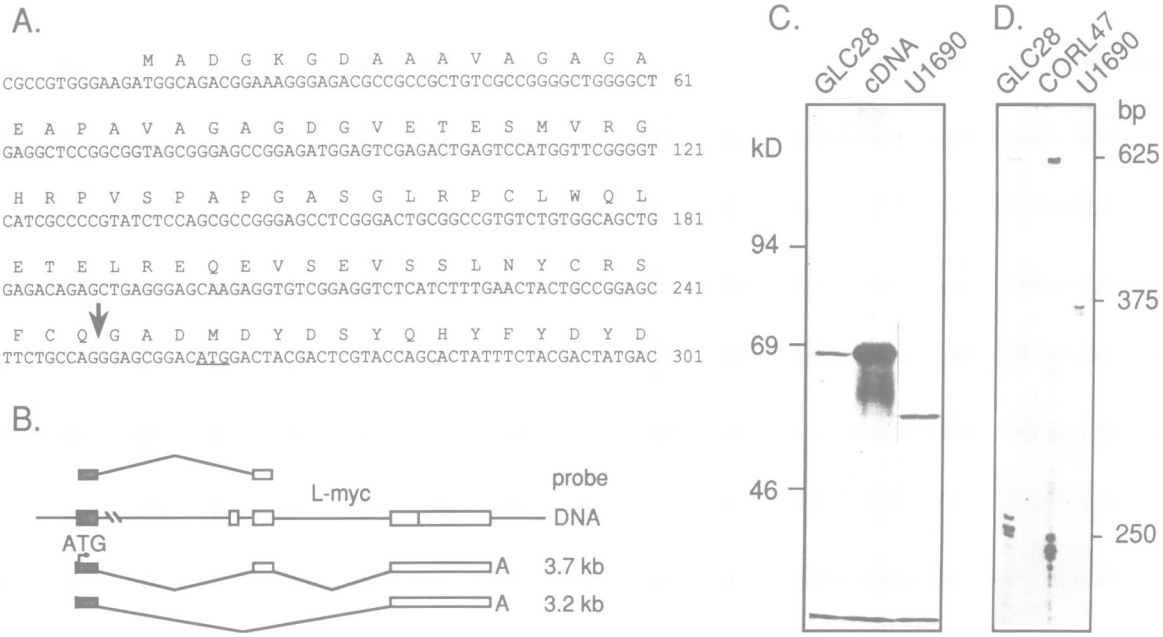

Fig. 3. Characterization of chimeric L-*myc* clones from a GLC28 cDNA library. **A.** Partial nucleotide sequence from cDNA clones G251 and G375 is shown with the deduced amino acid sequence above. The breakpoint is indicated by an arrow, and corresponds to nucleotide 796 in L-*myc* in Kaye *et al.* (1988); GenBank pr:hummyc31); the normal L-*myc* initiator codon is underlined. The sequence shown here has been deposited in the EMBL database (accession number X58480). **B.** A schematic diagram of the rearranged DNA and of the fused RNAs. The transcripts of 3.7 and 3.2 kb are formed by differential splicing as shown. The probe used in D is indicated at the top. **C.** *In vitro* translated protein from the fusion cDNA is compared with the normal (U1690) or large (GLC28) L-*myc* proteins. Prior to SDS−PAGE and autoradiography, samples were immunoprecipitated and treated with phosphatase. **D.** RNase mapping of polyadenylated RNA from the indicated cell lines with a probe shown schematically in B. The 625 and 250 bp protected bands represent the 3.7 and 3.2 kb fusion transcripts, respectively. The 385 bp band from the control U1690 cells represents L-*myc* exon II sequences.

abnormal transcripts show two irregularities when compared with normal expression of L-*myc* (Kaye *et al.*, 1988). They lack expression of the L-*myc* short form transcripts, which terminate in intron II of L-*myc*, and they contain L-*myc* transcripts, where the second exon is spliced. These abnormalities may be due to structural differences in the mRNAs with the novel upstream exon.

The novel mRNA encodes a 446 amino acid polypeptide and has a similar structure in GLC28 and CORL47

Sequence analysis of the novel 5′ region from several clones representing the 3.7 kb mRNA predicted a 446 amino acid (aa) fusion protein with 79 aa from the novel exon, 3 aa from the untranslated sequences of L-*myc* second exon, and 364 aa from the L-*myc* coding sequences (Figure 3A). It was confirmed that the polypeptide predicted from the cDNA clones represents the novel L-*myc* related protein detected in GLC28 and CORL47 by *in vitro* transcription and translation of the cDNA clones. The products of *in vitro* translation were compared with the immunoprecipitated and phosphatase treated protein from GLC28 cells (Figure 3C), and both polypeptides were found to have identical mobilities of 70 kd in SDS–PAGE.

Results from the immunoprecipitation analysis (Figure 1) and from Northern blotting (Figure 2) had indicated that the novel polypeptides and mRNAs were very similar in both GLC28 and CORL47 cells. In order to analyze this apparent similarity, RNase mapping was used to study L-*myc* mRNA structure in CORL47. A probe spanning the breakpoint in GLC28 protected identical bands of 625 and 250 bases in both cells. These two bands represent the two mRNA forms detected in Northern blotting. When mRNA from the control U1690 cells was used, only a single band of 375 bp representing sequences from L-*myc* exon II was seen. These results indicated that the mRNA junction between the novel sequences and L-*myc* was identical in both cell lines (Figure 3D).

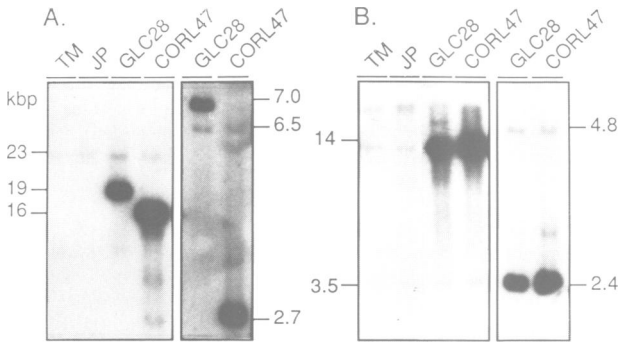

Fig. 4. DNA rearrangements of the novel sequences in cell lines with the fusion protein. **A**. Southern analysis with a probe from the novel sequences was carried out with *Eco*RI (left panel) or *Pvu*II (right panel) digested DNA from the indicated sources. JP and TM represent normal DNA. GLC28 and CORL47 DNAs contain rearranged, amplified bands of 19 and 16 kb, respectively. **B**. Southern analysis with an L-*myc* probe with DNA from indicated sources digested with *Nsi*I (left panel) or *Pvu*II (right panel); the normal L-*myc* is in the 14 kb fragment, and shows an amplification, but no rearrangement for GLC28 and CORL47. The 3.5 kb band corresponds to the L-*myc* pseudogene (DePinho *et al.*, 1987), which was consistently detected in our hybridizations.

The chimeric polypeptide results from dissimilar DNA rearrangements in GLC28 and CORL47

To investigate whether the novel 5′ sequence in GLC28 and CORL47 represents an alternative exon I of L-*myc* Southern blotting analysis was carried out using a probe from the novel 5′ cDNA sequence. Hybridization results indicated that this probe represents a single locus in the human genome (Figure 4A). However, the two cell lines expressing the large protein revealed distinct rearranged and amplified DNA bands in several digestions, suggesting that the novel mRNA and protein in GLC28 and CORL47 cells result from a DNA rearrangement placing a normally unrelated first exon upstream of L-*myc*.

The rearrangements occurred within 7 kb (GLC28) and 3 kb (CORL47) downstream of the exon present in the fusion mRNAs (Figure 3A) in a region of 4 kb, which may represent a breakpoint cluster region. The possible similarity of the breakpoints on the L-*myc* side, and their distance from L-*myc*, could not be determined in Southern analysis (Figure 4B), indicating that they have occurred at least 10 kb upstream of L-*myc*. The presence of a unique rearranged amplified restriction digestion fragment in both cases strongly suggests that the rearrangements have occurred before gene amplification.

The fusion cDNA is formed by L-myc and a novel gene named rlf

The novel 5′ cDNA sequences did not reveal a significant degree of homology with sequences in the databases searched (GenBank R66, EMBL R25, Swissprot R16). To investigate whether they represent a separately transcribed gene, a cDNA library from the K562 cell line was screened, and a clone hybridizing only to the novel sequences was isolated (Figure 5A). Preliminary characterization of this 1.2 kb clone confirmed that it contains a sequence identical to that present in the fusion mRNA of GLC28 and CORL47 followed by unknown sequences at the 3′ end. When this cDNA probe was used as a probe in Northern blotting, it detected a 7.0 kb mRNA in several cell lines (Figure 5B) and human embryonal tissues (data not shown) in addition to the fusion mRNAs of 3.2 and 3.7 kb in GLC28 and CORL47. Thus it is concluded that the cDNA probe represents a new transcribed gene, which we have named *rlf* (for **r**earranged **L**-*myc* **f**usion).

Discussion

Our studies show that similar chimeric proteins seen in two unrelated SCLC cell lines result from independent recombination events involving the same two genes, *rlf* and L-*myc*. It is interesting to note that these rearrangements could not have been previously detected in Southern analyses using L-*myc* probes due to the distance of the breakpoints from L-*myc*. However, the rearrangements are readily detectable with the *rlf* probe because the breakpoints are <7 kb from the *rlf* exon. Therefore we have attempted to use the *rlf* probe to investigate how common the *rlf*–L-*myc* fusion is in human lung cancer. In a primary analysis of a panel of human lung cancer DNAs we have already detected one rearrangement with the *rlf* probe (T.P.Mäkelä, M. Shiraishi and K.Alitalo, unpublished), and we are currently cloning L-*myc* upstream probes in order to confirm that the L-*myc* gene is also involved in this rearrangement. Since

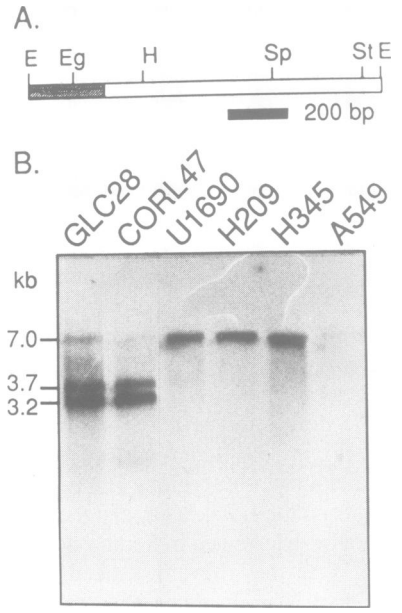

Fig. 5. The *rlf* gene is expressed as a 7.0 kb transcript. **A.** A K562 cDNA library was screened with *rlf* sequences from the chimeric clone, and a 1.2 kb cDNA clone containing 260 bp sequences from the chimera at the 5′ end (darker area) and 1 kb of normal sequences was obtained. E, *Eco*RI; Eg, *Eag*I; H, *Hin*dIII; Sp, *Sph*I; St, *Stu*I. **B.** Polyadenylated mRNA from the indicated cell lines was analyzed by Northern blotting with the cDNA probe from A.

the location of the breakpoint joining these two genes might vary considerably also with respect to the *rlf* gene (due to a long intron of >15 kb in *rlf*; unpublished data of the authors), we are also developing new methods to measure the prevalence of *rlf*−L-*myc* fusions in human cancer more reliably. One attractive approach would be to use a PCR cDNA assay with primers from both sides of the *rlf*−L-*myc* junction in the chimeric mRNA.

In both of the cell lines used in this study the *rlf*−L-*myc* fusion gene has also been involved in a DNA amplification. Since hybridization analyses using an *rlf* probe only show a single rearranged, amplified band in restriction mapping, it can be concluded that the genomic amplifications in these cell lines must have occurred after the *rlf*−L-*myc* fusion. It is not known whether gene amplification has occurred soon after the rearrangement, or if it represents a later event associated with the progression of these SCLCs. Analysis of a larger material of tumors should reveal examples of single-copy *rlf*−L-*myc* rearrangements if they occur independently of DNA amplification. In fact, this appears to be the case with the tumor DNA which we have found to display an abnormal single-copy *rlf* hybridization pattern.

The *rlf*−L-*myc* gene fusion could lead to oncogenic activation of the *rlf* gene by at least two distinct mechanisms. Firstly, the rearrangements cause a switch from the transcriptional elements of L-*myc* to those of *rlf*, resulting in deregulation of the normally strictly controlled L-*myc* expression. A similar promoter switch has been suggested to be the activating mechanism of c-*myc* in a woodchuck liver tumor, where the *hcr* locus fuses upstream of c-*myc* (Etiemble *et al.*, 1989). Alternatively, the alteration of the amino terminus of L-*myc* could affect the activity of substrate specificity of the L-*myc* protein. Regarding the latter alternative, it is worth noting that the transforming avian v-*myc* proteins contain viral sequences at their amino termini (Frykberg *et al.*, 1987; Symonds *et al.*, 1989).

An interesting possibility is that the *rlf*-encoded sequences modulate the function of the fusion protein in a specific way. Such a mechanism has been suggested as the basis of oncogenic activation in the translocation and fusion between transcription factors E2A and prl (Kamps *et al.*, 1990; Nourse *et al.*, 1990). Accordingly, it is also known that the leukemia-specific bcr−abl fusion protein has an enhanced tyrosine kinase activity compared with the normal c-abl protein (Lugo *et al.*, 1990).

In addition to the bcr−abl and prl−E2A, only three other fusion proteins have been identified in human tumors: the tropomyosin−*trk* from a colon carcinoma (Martin-Zanca *et al.*, 1986), the t(15;17) *myl*−retinoic acid receptor α fusion in acute promyelocytic leukemia (Borrow *et al.*, 1990; deThé *et al.*, 1990), and the H4−*ret* (PTC) protein frequently found in thyroid papillary carcinomas (Grieco *et al.*, 1990). The H4−*ret* fusion apparently does not result from a translocation between two chromosomes, but instead from an intrachromosomal rearrangement, since both genes involved map to human chromosome 10 band q11.2 (Ishizaka *et al.*, 1989; Grieco *et al.*, 1990). Similarly, our results indicate that both the L-*myc* and *rlf* genes reside close to each other in germline DNA on chromosome 1 (T.P.Mäkelä, J.Kere, R.Winqvist and K.Alitalo, manuscript in preparation).

The *rlf*−L-*myc* fusion protein represents the first example of a chimeric *myc* oncoprotein in human cancer. This may represent a novel activation mechanism for the *myc* family of proto-oncogenes.

Materials and methods

Cell lines

All cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum and antibodies. GLC28 cells were kindly given by Dr Charles Buys, U1690 cells by Dr Jonas Bergh, NCI-H209 and NCI-H345 by Dr Adi Gazdar and CORL88 by Dr Pamela Rabbitts. CORL88 and CORL47 cells have been characterized by Baillie-Johnson *et al.* (1985).

Immunoprecipitation

Metabolic labeling, immunoprecipitation, alkaline phosphatase treatment and SDS−PAGE were performed as described (Mäkelä *et al.*, 1989). The pan-myc antibody precipitates both the large and small form of the L-*myc* protein (Saksela *et al.*, 1989), whereas the NCM II 274 monoclonal antibody (kindly given by Dr Naohiko Ikegaki) precipitates exclusively the large L-*myc* protein (Ikegaki *et al.*, 1989).

cDNA cloning and characterization

Five micrograms of polyadenylated RNA from cell line GLC28 was used to produce double-stranded complementary DNA according to standard protocols using the Amersham cDNA synthesis kit. After *Eco*RI linker ligation, cDNA was size fractionated in low-melting point agarose, and products > 1.8 kb were ligated into λgt10. Differential screening of $\sim 8 \times 10^5$ recombinant phage with L-*myc* exon II and exon III probes resulted in 20 positive clones, which were initially analyzed for common restriction sites. Relevant cDNA inserts were subcloned into pGEM3Zf(+) (Promega) and sequenced as double-stranded DNA using Sequenase (United States Biochemicals). Screening of the K562 cDNA library (Grosveld *et al.*, 1986; kindly given by Dr Gerard Grosveld) was performed with a probe obtained with the polymerase chain reaction from cDNA clone G251 representing the novel sequences.

In vitro transcription and RNase protection assays

Antisense RNA representing the fusion cDNA was transcribed *in vitro* from pGEM3Zf(+) as recommended by the supplier. For RNase mapping probes, [α-^{32}P]UTP was added to the reaction. The probe was synthesized from cDNA clone G251, and spans from nucleotide 1 in Figure 3A to nucleotide

1170 in Kaye *et al.* (1988). RNase protection was performed as described (Mäkelä *et al.*, 1987).

In vitro translation

The full-length cDNA construct was assembled from two partial clones, and *in vitro* translation from *in vitro* transcribed RNA (see above) was performed in the presence of [^{35}S]methionine as recommended (Promega). Alkaline phosphatase treatment and gel electrophoresis were performed as described (see above).

Hybridization analysis of RNA and DNA

RNA and DNA isolation, and Northern and Southern blotting analyses were done according to standard procedures (Maniatis *et al.*, 1982).

Computer analysis

For DNA and protein sequence analyses, the University of Wisconsin Genetics Computer Group software package (Devereux *et al.*, 1984) was used on a VAX8800 computer (Digital Equipment Corporation). Predicted protein sequence comparisons were made to Swissprot release 16, and DNA sequences to GenBank release 66 and EMBL release 25.

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