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Identification of Rauscher murine leukemia virus-specific mRNAs for the synthesis of *gag*- and *env*-gene products

(protein synthesis/oocytes/radioimmunoprecipitation/gel electrophoresis)

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ABSTRACT **Polyadenylylated mRNA isolated from cells infected with Rauscher murine leukemia virus was fractionated by centrifugation in a denaturing sucrose gradient into different sizes. Each RNA fraction was injected into oocytes of *Xenopus laevis* and the virus-specific products were analyzed by immunoprecipitation with polyvalent and monospecific antisera against polypeptides of Rauscher murine leukemia virus, and then by gel electrophoresis and scintillation autoradiography. It was shown that a 35S mRNA species directs the synthesis of a precursor of the internal or group-specific antigens of the virion (the *gag*-gene products). A 22S mRNA species directs the synthesis of two viral envelope polypeptides and their precursor polypeptide (*env*-gene products). The results indicate that the *gag*- and *env*-related polypeptides of Rauscher murine leukemia virus are synthesized uncoordinately and provide evidence for open and closed cistrons on the virus-specific mRNAs.**

The structural polypeptides of the murine leukemia viruses and, presumably, also those of all other RNA tumor viruses are subgene products derived from the primary translation products (precursor polypeptides) of the *gag*- and the *env*-gene (for a review see ref. 1). In our studies with JLS-V9 cells, infected with Rauscher murine leukemia virus (R-MuLV), we have identified a glycosylated polypeptide with a molecular weight of 82,000 (*env*-pr82) as the precursor of two polypeptides of the viral envelope, gp69/71 and p15(E) (2, 3). The polypeptide p12(E) (designated p12 in ref. 3) shares chymotryptic peptides with p15(E); therefore p12(E) is also derived from the *env*-precursor polypeptide (our unpublished results and ref. 4). In the same studies (2, 3), we recognized two mutually related polypeptides with molecular weights of 75,000 (called *gag*-pr75) and 65,000 (called *gag*-pr65) as the precursors of the internal virion polypeptides p30 and p15. Recently, Barbacid *et al.* (5) demonstrated that the two other internal polypeptides, p12 and p10, are also derived from the precursor of p30 and p15. All these data are consistent with those reported by several other authors (4, 6–9).

The existence of virus-specific mRNA species of different size classes (10) suggests that the synthesis of *gag*- and *env*-precursor polypeptides is programmed by different mRNAs rather than by one polycistronic mRNA. In agreement with this suggestion, it was shown that each virus-specific mRNA species mediated the synthesis of its own virus-specific product(s) in a rabbit reticulocyte cell free system (11). Of the two virus-specific products formed in this system under the direction of the 35S mRNA fraction, one comigrated during sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis with the *gag*-pr65 mentioned above, the other one with *gag*-pr72, a *gag*-related polypeptide found in infected cells in the presence of the arginine analog canavanine (3). Similar products were found in various protein synthesizing systems programmed with RNA isolated from virions of RNA tumor viruses and identified as *gag*-related products (12–17). In none of these experiments with virion RNA, however, could *env*-related products be detected.

In addition to these results obtained with genome size virus-specific RNA (35S) (whether isolated from virions or as polyadenylylated mRNA from infected cells), two observations described in our previous study are noteworthy: (*i*) the 22S mRNA fraction isolated from cells infected with R-MuLV mediated the synthesis of a virus-specific 70,000-dalton polypeptide in a cell-free system from reticulocytes (11), a polypeptide of the same size as the *env*-related product formed in infected cells when glycosylation is inhibited (ref. 9 and W. J. M. van de Ven, unpublished); (*ii*) in an endogenous cell-free system derived from cells infected with R-MuLV, free polyribosomes containing 35S RNA as the main virus-specific mRNA produced *gag*-products only whereas the membrane-bound polyribosomes gave rise to both *gag*- and *env*-products and contained 22S virus-specific mRNA in addition to the 35S RNA (11). These results prompted us to localize the *gag*- and *env*-messenger activity among the heterologous virus-specific mRNAs.

MATERIALS AND METHODS

Cells and Virus. The JLS-V9 cell line derived from bone marrow cells of Balb/c mice infected with and producing R-MuLV was grown in Eagle's basal minimal essential medium supplemented with 10% calf serum (18). Labeling of JLS-V9 cells and preparation of cell lysates for immunoprecipitation was described elsewhere (3).

Isolation of Polyribosomes. For the preparation of total polyribosomes, cells were treated and subsequently harvested as described before (11). After the cells (2–3 ml) were washed and swollen in hypotonic buffer (11), they were disrupted by 10 strokes of a Dounce homogenizer (B pestle) in 12 ml of lysis buffer (50 mM Tris·HCl, pH 7.4/$Mg(Ac)_2$, 5 mM/KAc, 150 mM/Nonidet P-40, 1% (vol/vol)/sodium deoxycholate, 0.5% (wt/vol)/dithiothreitol, 2 mM/and 100 μg/ml of an RNase inhibitor from bovine eye-lens purified on DEAE cellulose (obtained from H. Bloemendal). Nuclei and cell debris were removed by centrifugation for 4 min at top speed (20,000 rpm) in a Ti50 rotor (Beckmann). The supernatant was layered onto 1.0 ml of 2 M sucrose in 20 mM Tris·HCl, pH 7.4/KAc, 120 mM/$Mg(Ac)_2$, 5 mM/and 2 mM dithiothreitol, and polyribosomes were collected by centrifugation for 2.5 hr at 56,000 rpm in an SW 56 rotor (Beckmann).

Isolation and Fractionation of mRNA. Polyadenylylated mRNA was isolated from total polyribosomes by affinity chromatography on oligo(dT)-cellulose as described (11) with

Abbreviations: R-MuLV, Rauscher murine leukemia virus; $NaDodSO_4$, sodium dodecyl sulfate; *gag* and *env* are each viral genes.

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the following modifications: polyribosomes were incubated in buffer A for 10 min at 37° with Pronase(RNase-free), 0.5 mg/ml and $NaDodSO_4$ was omitted from column buffer B. mRNA was fractionated by centrifugation in a denaturing sucrose gradient into various sizes as described in the legend to Fig. 1.

Antisera. Antisera raised against R-MuLV polypeptides and used for immunoprecipitation were described elsewhere (3). We observed no crossreactivity among the monospecific antisera used in this study; a slight coprecipitation, however, of gp69/71, p15(E), and p12(E) was observed in immunoprecipitation reactions with antiserum against gp69/71 and antiserum against both p15(E) and p12(E) (designated anti-p15(E), p12 serum in ref. 3) due to an association of these viral envelope polypeptides (3). The antiserum against p15(E), p12(E) was raised against a mixture of p15(E) and p12(E) (3); moreover, it is now known that p15(E) and p12(E) contain common amino acid sequences (D. Van Zaane, unpublished results, and ref. 4). To rule out contamination with antibodies against reverse transcriptase, we tested the antisera against R-MuLV, gp69/71 and p30 for their capacity to inhibit the enzyme activity. The sera did not react in this test (not shown) and, therefore, they will probably not immunoprecipitate reverse transcriptase.

RESULTS

Virus-Specific mRNA Activity of Total Polyadenylylated RNA from Infected Cells. Translation of 35S RNA isolated from R-MuLV in oocytes of *Xenopus laevis* has clearly demonstrated that this RNA preparation directs the synthesis of *gag*-pr65 only (15). In a preliminary experiment with these oocytes, we observed that a mixture of virus-specific mRNAs of different size classes (14 S, 22 S, and 35 S) isolated from JLS-V9 cells infected with R-MuLV mediated the synthesis of the *gag*-gene product, *gag*-pr65, as well as the *env*-gene products, *env*-pr82, gp69/71, and p15(E) (not shown). These observations indicate that at least two separate mRNA functions are present among the population of virus-specific mRNAs: a *gag*-specific and an *env*-specific mRNA. To identify both mRNA activities, we repeated this experiment with fractionated mRNA.

Virus-Specific mRNA Activity of Fractionated mRNA. The fractionation of mRNA from infected cells was performed by sucrose gradient centrifugation in the presence of formamide as a denaturing agent. The sedimentation pattern of virus-specific mRNA in the gradient was determined by hybridization with DNA complementary to R-MuLV-RNA (Fig. 1). The results are essentially the same as those reported before (11) and the virus-specific mRNA species of 14 S, 22 S, and 35 S can be distinguished. Oocytes of *Xenopus laevis* were injected with RNA from each fraction and incubated with L-[^{35}S]methionine. The newly synthesized virus-specific polypeptides were separated from the large excess of JLS-V9-specific polypeptides and endogenous oocyte polypeptides by immunoprecipitation with anti-R-MuLV serum; this serum reacts with both *gag*- and *env*-related products (3). The immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and scintillation autoradiography (Fig. 2). Three polypeptides, two in the 50,000 dalton region and one with a very high molecular weight, were precipitated in a control experiment with oocytes injected with water (Fig. 2, *slot 5*) as well as in all other analyses of injected oocytes (Fig. 2, *slots 6–19*). Apparently these polypeptides are not virus-specific and, therefore, will be ignored in further discussions. A number of virus-specific polypeptides can be recognized which comigrate with the R-MuLV-specific precursor polypeptides or with the mature virion polypeptides

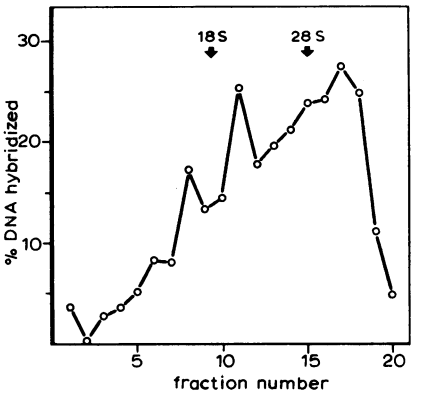

FIG. 1. Sucrose gradient centrifugation of virus-specific mRNAs from JLS-V9 cells infected with R-MuLV. Polyadenylylated mRNA (32 μg) isolated as described in *Materials and Methods* section was denatured at 56° for 3 min with formamide, 85% (vol/vol) in 20 mM Tris·HCl, pH 7.4/EDTA, 1 mM/and 0.5% $NaDodSO_4$, (wt/vol). After dilution with one volume of TNE (10 mM Tris·HCl, pH 7.4/NaCl, 100 mM/EDTA, 1 mM) the RNA preparation was layered onto an isokinetic sucrose gradient in TNE containing 50% (vol/vol) formamide [C_t (top) = 5% (wt/vol), C_h (high) = 34.4% (wt/vol), V_m (min) = 12.9 ml]. The tubes were spun in a SB 283 rotor (IEC) for 13 hr at 4° at 41,000 rpm. RNA from each fraction was precipitated with alcohol after addition of 10 μg of 28S RNA from calf eye-lens ribosomes as a carrier. The relative amount of virus-specific RNA was determined in each fraction by hybridization with DNA complementary to R-MuLV RNA (10).

immunoprecipitated from infected cells (Fig. 2, *slots 20* and *21*).

The autoradiogram of virus-specific translation products (Fig. 2) suggests a sharp separation of virus-specific mRNA, which is in contrast with the diffuse hybridization pattern depicted in Fig. 1. This may be explained by the nonlinear blackening of the film during scintillation autoradiography and by the unknown representation of the viral RNA in the cDNA probe used for hybridization.

Identification of the *gag*-mRNA. In agreement with our previous study in which we used a cell-free system from reticulocytes (11), the 35S mRNA species (Fig. 1, fraction nos. 17 and 18) directed the synthesis of a 65,000-dalton polypeptide (Fig. 2, *slots 17* and *18*). Fig. 3 *I–K*, shows that the 65,000-dalton polypeptide synthesized in oocytes can be precipitated with antiserum against p30 as well as with antiserum against p15 but not with antiserum to gp69/71. Incidentally, it should be noted here that the antiserum against gp69/71 precipitates a polypeptide (about 32,000 daltons) from oocytes injected either with the various RNA fractions (Fig. 3*E*, *F*, and *K*) or with water (Fig. 3*L*), indicating that this polypeptide is an endogenous oocyte product. We conclude that the 65,000-dalton polypetide is similar if not identical to the *gag*-gene product, *gag*-pr65, found in infected cells. This conclusion is consistent with a reverse experiment carried out by Mueller-Lantzsch (21) showing that the polyribosomes, taken from cells infected with Moloney murine leukemia virus, that were immunoprecipitated with antiserum against p30 contained 35S RNA as the main virus-specific RNA species.

Identification of the *env*-mRNA. The 22S mRNA fraction (Fig. 1, fraction no. 11) mediates the synthesis of three polypeptides (Fig. 2, *slot 11*) which were identified as the *env*-gene products *env*-pr82, gp69/71, and p15(E): they comigrated with the *env*-gene products found in infected cells (Fig. 2, *slots 20* and *21*); the newly synthesized polypeptide comigrating with *env*-pr82 could be immunoprecipitated with antiserum against gp69/71 and antiserum against p15(E), p12(E), whereas those

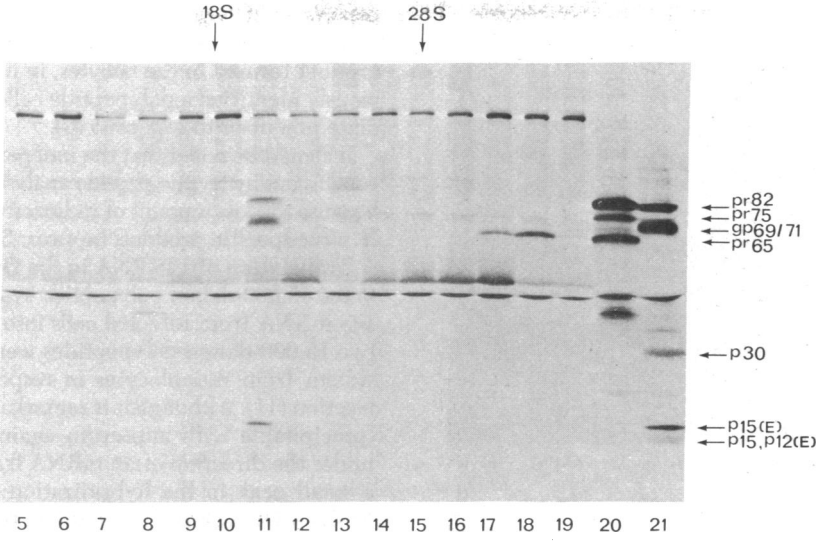

FIG. 2. Radioimmunoprecipitation analysis with antiserum against R-MuLV of the virus-specific products synthesized after injection of oocytes of *Xenopus laevis* with fractionated polyadenylylated RNA from JLS-V9 cells infected with R-MuLV. RNA precipitated from each fraction of the gradient shown in Fig. 1 was dissolved in 10 μl of H_2O; 0.5 μl was injected into 20 oocytes (19). The oocytes were incubated for 20 hr at 20° with 25 μCi of L-[^{35}S]methionine (specific activity, 329 Ci/mmol; from The Radiochemical Centre, Amersham, England) and, subsequently, homogenized in 1.25 ml of immunoprecipitation buffer (3). The homogenate was cleared by centrifugation for 10 min at 220,000 × *g* in a Ti50 rotor (Beckman). Virus-specific polypeptides from the supernatant were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (11) and scintillation autoradiography (20) after indirect immunoprecipitation with antiserum against R-MuLV (2, 3). *Slots 6–19* correspond with the fraction numbers of the gradient shown in Fig. 1. *Slot 5*: (control) (immunoprecipitation analysis with antiserum against R-MuLV of products synthesized after injection of 0.5 μl of H_2O into 20 oocytes). *Slot 20*: virus-specific polypeptides immunoprecipitated with antiserum against R-MuLV from infected JLS-V9 cells treated for 15 min with a ^{14}C-labeled amino acid mixture (3). *Slot 21*: the same as slot 20 except that the radioactivity was chased for 8 hr with an excess of unlabeled amino acids (3).

comigrating with gp69/71 and p15(E) were immunoprecipitated with the corresponding antisera (Fig. 3*F* and *G*). Fig. 3*H* shows that the *gag*-specific antiserum against p30 does not precipitate translation products of 22S mRNA.

Immunoprecipitable Translation Products of the 14S and 25S mRNA Fractions. The 45,000-dalton polypeptide formed after injection of 14S RNA (Fig. 1, fraction no. 8) and immunoprecipitated with the antiserum against R-MuLV (Fig. 2, *slot 8*) was not precipitated with the monospecific antiserum against p30, antiserum against p15, or antiserum against gp69/71 (Fig. 3*C–E*). The nature of this product remains unknown; we cannot exclude the possibility that it is coded for by a host 14S mRNA. Finally, the 25S mRNA fraction (Fig. 1, fraction no. 13) directs the synthesis of a minor polypeptide (85,000–90,000 daltons) which was precipitated with antiserum against R-MuLV (Fig. 2, *slot 13*) but not with antiserum against p30 or antiserum against gp69/71 (not shown). This polypeptide could represent a host-specific product from JLS-V9 cells recognized by the antiserum against R-MuLV. Some unidentified polypeptides in the same size range were precipitated by the antiserum against R-MuLV when 25–28S mRNA from infected cells was translated in the reticulocyte system (11).

DISCUSSION

Open and Closed Cistrons on Oncornaviral mRNA. In 1974, Baltimore (22) proposed four "genetic elements" for the avian sarcoma viruses: *gag* (for the precursor polypeptide of the major core protein), *env* (for the major envelope protein), *pol* (for the RNA dependent DNA polymerase) and *onc* (for cell transformation). He discussed them "as if they were genes constituting most of the genome of an RNA tumor virus." Since that time, all known structural polypeptides of the murine leukemia viruses could be assigned to either the *gag*- or the *env*-gene. These two genes, therefore, contain "subgenes"; their primary translation products are precursor polypeptides that are cleaved into subgene products.

Our present results show that each of the two genes, *gag* and *env*, is expressed by translation of its own specific mRNA. Such virus-specific mRNAs although functionally monocistronic may apparently overlap other genes and, therefore, be structurally polycistronic. This is obvious for the genome size 35S *gag*-mRNA and probable for the 22S *env*-mRNA with its estimated molecular weight of 1.2×10^6 (10). Similar conclusions follow from hybridization data on oncornaviral mRNAs of different size classes (10, 23–25). It is tempting to speculate that as a rule the open (i.e., translated) cistron would be the 5′-terminal one. Otherwise a more complicated model would be required to explain the recognition of the open initiation site. For the *gag*-mRNA, this speculation would be in agreement with the probable gene order proposed for the avian RNA tumor viruses, 5′-*gag-pol-env-onc*-poly(A)-3′ (26). By assuming the same gene order the *env*-mRNA would then have the structure 5′-*env-onc*-poly(A)-3′.

Much less is known on the expression of *pol* and *onc*; the latter gene although identified from mutants of murine sarcoma viruses (27) is still hypothetical in the case of leukemia viruses. If *pol* and *onc* also have separate mRNAs the model predicts the following structures: 5′-*pol-env-onc*-poly(A)-3′ and 5′-*onc*-poly(A)-3′.

Very Large Virus-Specific Polypeptides. The concept of separate mRNAs for the *gag*- and the *env*-gene agrees with data on the uncoordinate expression of p30 and gp69/71 in cells harboring endogenous or defective RNA tumor viruses (28, 29). Likewise, some recent experiments in which the initiation of protein synthesis was influenced by high salt conditions (30) indicated that different sites for the initiation of protein synthesis are operative in the expression of the *gag*- and the *env*-gene of nondefective mouse mammary tumor virus. From these experiments and from the results described in this paper, we

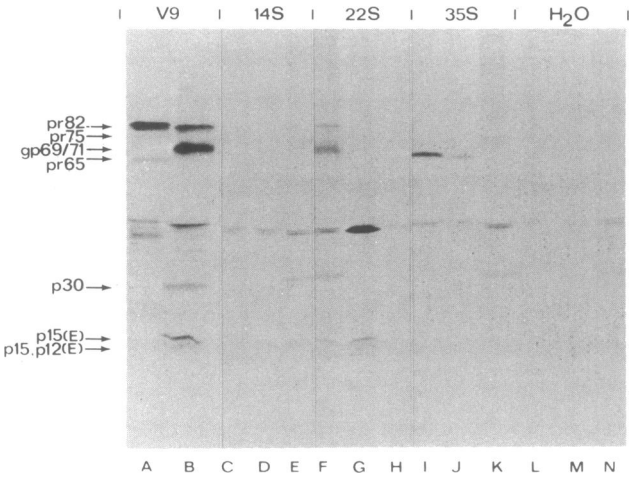

FIG. 3. Monospecific antisera identification of R-MuLV-specific products formed in oocytes of *Xenopus laevis* after injection with mRNA fractions from JLS-V9 cells infected with R-MuLV. Technical details and fraction numbers of mRNA are as described in the legend to Fig. 2. Lysates of oocytes injected with 14S mRNA (fraction no. 8; *slots C–E*), 22S mRNA (fraction no. 11; *slots F–H*) and 35S mRNA (fraction no. 18; *slots I–K*) were analyzed with monospecific antisera. The following monospecific antisera were used: antiserum against p30 (*slots C, H, I,* and *L*); antiserum against p15 (*slots D, J,* and *M*); antiserum against gp69/71 (*slots E, F, K,* and *N*); antiserum against p15(E), p12(E) (*slot G*). As a control, similar analyses were performed with oocytes injected with H_2O (*slots L–N*) and as a reference the virus-specific polypeptides from infected cells are shown (*slots A* and *B*). (*Slot A*) Virus-specific polypeptides immunoprecipitated with antiserum against R-MuLV from lysates of JLS-V9 cells infected with R-MuLV treated for 15 min with ^{14}C-labeled amino acids. (*Slot B*) The same as *slot A* except that the radioactivity was chased for 8 hr with an excess of unlabeled amino acids.

conclude that translation of the genome of RNA tumor viruses into one giant precursor polypeptide (250,000–300,000 daltons) as found for picornaviruses (31, 32) is unlikely or, at least, is not the principal pathway for the synthesis of the virus-specific polypeptides. Some authors have noticed varying amounts of large virus-specific polypeptides (>100,000 daltons) in infected cells (8, 9); their physiological significance, however, remains obscure.

Synthesis of the *env*- and *gag*-Products in the Oocyte System. In our previous study, a 70,000-dalton polypeptide was synthesized in a cell-free system from reticulocytes after the addition of 22S mRNA isolated from cells infected with R-MuLV (11). Presumably, this polypeptide was the protein moiety of the glycosylated *env*-pr82 (3) because a 70,000-dalton polypeptide was also found in infected cells when glycosylation was inhibited with D-2-deoxyglucose or cytochalasin B (ref. 9; W. J. M. van de Ven, unpublished results). In oocytes of *Xenopus laevis*, however, the *env*-products were apparently glycosylated because they comigrated with *env*-pr82 and gp69/71. The synthesis of *env*-pr82 was also observed in an endogenous cell-free system from infected cells containing polyribosomes still attached to membranes (11). It is generally accepted (33) that an enzyme system associated with the endoplasmatic reticulum recognizes signals for the glycosylation of nascent polypeptides. This enzyme system is apparently well conserved during the evolution of the vertebrates. The same seems to be true for another posttranslational activity, the cleavage of precursor polypeptides, because in addition to *env*-pr82 the subgene products gp69/71 and p15(E) are also formed in the amphibian oocytes. The *gag*-related polypeptides were less well represented in oocytes although in some experiments, a partial processing of *gag*-pr65 was observed (not shown). In the experiments presented here, *gag*-pr65 was the sole *gag*-specific product formed in the oocytes; in infected cells, we had previously identified a polypeptide called *gag*-pr75 as the immediate precursor to *gag*-pr65 (3).

It should be noted that the independent confirmation of our conclusions by tryptic peptide analysis has not been undertaken because the low amount of radioactivity that was incorporated in virus-specific products (approx. 500 cpm/20 oocytes).

Translation of 14S RNA in the Oocyte System. In contrast to the 45,000-dalton polypeptide synthesized after injection of 14S mRNA from infected cells into oocytes, one 30,000- and two 15,000-dalton polypeptides were produced in a cell-free system from reticulocytes in response to the same mRNA fraction (11). Although it is remarkable that all these products (precipitable with antiserum against R-MuLV) are formed under the direction of an mRNA fraction that coincides with a small peak in the hybridization profile obtained with R-MuLV-cDNA (Fig. 1), the origin, the nature, and the possible relationship between these polypeptides are not yet clear.

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