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Monoclonal antibodies to human urokinase identify the single-chain pro-urokinase precursor

(plasminogen activator/*in vitro* synthesis/immunoblotting/immunofluorescence/fibrinolysis)

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ABSTRACT **Monoclonal antibodies have been obtained that recognize either the A or B chain of human urinary urokinase. These antibodies identify human urokinase-producing cells and the product of urokinase mRNA. Anti-urokinase monoclonal antibodies precipitate an ≈54,000-dalton protein synthesized *in vitro* in a rabbit reticulocyte cell-free system. This pro-urokinase appears to be the precursor of both A and B chains of human urinary urokinase. Urokinase mRNA in human kidney constitutes only 0.1% or less of total poly(A)$^+$ RNA.**

Plasminogen activators are proteases that activate plasminogen to plasmin. This reaction is of great importance in fibrinolysis; in addition, plasminogen activator appears to play an important role in a variety of processes in which extracellular proteolysis is involved, like terminal differentiation and tissue remodeling (1). Induction of plasminogen activators has been observed both in physiological systems (2) and in *in vitro* cell culture systems. Different agents are effective *in vitro:* among others are tumor promoters (3), retinoic acid (4), tumor virus transformation (5), and DNA-damaging reagents (6).

At least two types of plasminogen activators have been isolated, the urinary activator (also called urokinase) and the tissue activator. The urinary activator urokinase is a protein of ≈54,000 daltons, which is found to be made up of two disulfide-linked chains: A, of ≈18,000 daltons; and B, of ≈33,000 daltons. The amino acid sequence of both chains is completely known (7–9). The tissue activator is a single-chain protein of 70,000 daltons and is unrelated to urokinase immunologically (10).

In view of the interest in studying the mechanisms regulating the synthesis of these enzymes and their role in differentiation and tissue remodeling, it would be desirable to study the synthesis of urokinase and its regulation at a molecular level both *in vivo* and *in vitro*. Towards these goals, we developed monoclonal antibodies (mAbs) against human urinary urokinase and used them to identify a single-chain biosynthetic precursor in a cell-free protein-synthesizing system directed by human kidney total poly(A)$^+$ RNA. These antibodies are available to other investigators for research.

MATERIALS AND METHODS

Materials. All chemicals used were of analytical grade. The following special materials were used: protein A-Sepharose (Pharmacia); sheep anti-mouse Ig antiserum conjugated to horseradish peroxidase (New England Nuclear); *o*-phenylenediamine (Fluka); class-specific rabbit anti-mouse Ig antisera (Nordic, Lausanne, Switzerland); fluorescein-conjugated F(Ab')$_2$ fragment of sheep anti-mouse Ig (New England Nuclear). Highly-purified 54,000-dalton urokinase (120,000 units/mg) was provided by L. Guzzi, and rabbit anti-urokinase antiserum was from L. Primi (Lepetit Research Laboratories, Milan, Italy).

Cell Lines and Media. The nonproducer mouse myeloma line X63-Ag8.653 (11) was used for the fusion experiments. Human kidney carcinoma line A1251 was obtained by S. Aaronson (Bethesda, MD). Diploid human fibroblasts F13 were obtained from A. Fusco (Naples, Italy). Rat-2 thymidine kinase-negative (TK$^-$) cells were obtained from M. Fried (London, U.K.). All cells were grown in Dulbecco-modified Eagle's medium (DME medium) (GIBCO) supplemented with 10% calf serum.

Production of mAbs. Female BALB/c mice were immunized by injecting into the footpad of the animal 100 μg of highly purified human urinary urokinase in complete Freund's adjuvant (diluted 1:1) at day −18. At day −3 the same amount of urokinase in 100 μl of phosphate-buffered saline (P_i/NaCl) was injected intraperitoneally. To obtain mAbs (12), at day 0 spleen cells (5×10^7) of the immunized mice were fused to 2×10^7 mouse myeloma X63-Ag8.653 cells. The fusion was carried out with polyethylene glycol 1000, and fusion material was plated into five different 24-well Costar plates containing 5×10^4 mouse macrophages (feeder layer) in DME medium containing 10% fetal calf serum and HAT (0.1 mM hypoxanthine/0.4 μM aminopterin/16 μM thymidine) (13). Supernatants (50–100 μl) of the growing hybrids were tested every 2–3 days by an enzyme-linked immunosorbent assay (ELISA) for production of anti-urokinase mAbs. In one single experiment, 98 antibody-producing hybrid clones were obtained, 8 of which were positive in the anti-urokinase ELISA. Cells from positive wells were repeatedly cloned and grown into the peritoneum of a BALB/c × C57BL/6 mouse by standard procedures. mAbs were purified from the ascites fluids by precipitation with 50% ammonium sulfate, dialyzed with 20 mM Tris·HCl/20 mM NaCl, pH 7.8, followed by affinity chromatography on protein A-Sepharose or UK-Sepharose.

ELISA Methods to Recognize Anti-Urokinase mAbs. A 96-well polyvinyl chloride microtiter plate (Dynatech, Alexandria, VA) was coated with urokinase and blocked with bovine serum albumin by standard procedures. To screen for anti-urokinase mAbs, 50–100 μl of the supernatant of hybridoma cultures was placed into the urokinase-coated microtiter plates and incubated for 1 hr at room temperature, washed five times with P_i/NaCl, and allowed to react with a 1:500 dilution of sheep anti-mouse Ig antiserum conjugated with horseradish peroxidase. After a thorough washing, plates were incubated with 50 μl of *o*-phenylenediamine (1 mg/ml) in 0.1 M citrate buffer, pH 5/1.75 mM H_2O_2 per well.

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Abbreviations: mAb(s), monoclonal antibody(ies); ELISA, enzyme-linked immunosorbent assay; TPA, phorbol 12-tetradecanoate 13-acetate.

After 30 min the reaction was stopped with 10% $NaDodSO_4$ and the optical density was read at 450 nm.

Class Specificity of mAbs. Class specificity of anti-urokinase mAbs was determined by Outcherlony's double immunodiffusion in agar or by ELISA.

Purification of 18,000- and 33,000-Dalton Urokinase Chains. Highly purified human urinary urokinase was dissolved in 0.05 M phosphate buffer (pH 7.0) and incubated for 120 hr at room temperature. The sample was then applied onto a Sephadex G-100 superfine column, equilibrated and eluted with 0.05 M phosphate buffer/0.2 M sodium chloride. Three peak fractions were obtained, concentrated, and analyzed on $NaDodSO_4$/polyacrylamide electrophoresis gel (12.5% acrylamide). Each peak gave a single band of 54,000, 33,000, or 18,000 daltons, respectively.

Immunoblotting of Urokinase and Purified Urokinase Chains. Urokinase or a purified chain (6–7 μg) was run onto a 12.5% $NaDodSO_4$/polyacrylamide electrophoresis gel. Transfer on nitrocellulose paper (Bio-Rad) was carried out at 100 mA for 18 hr in 25 mM Tris·HCl/182 mM glycine/20% (wt/wt) methanol, pH 8.3, at 4°C. After transfer half of the nitrocellulose strip was stained with amino black and destained with methanol/acetone/H_2O. The other half of each strip was washed in P_i/NaCl for 30 min, incubated for 1.5–2 hr with 3% bovine serum albumin in P_i/NaCl with gentle swirling. Reaction with mAbs was carried out in 0.3% bovine serum albumin in P_i/NaCl for 3 hr at 37°C. Strips then were washed carefully with 0.025% Tween in P_i/NaCl for 30 min with changes and were incubated for 1 hr at 37°C with goat-anti-mouse IgM-peroxidase in P_i/NaCl for mAb 52.C7 or with goat anti-mouse IgG-peroxidase for mAbs 65.F4 and 105.IF10. After a careful washing with 0.025% Tween in P_i/NaCl, the peroxidase substrate 3-amino-4-ethylcarbazole was added (10 mg in 2.5 ml *N,N*-dimethylformamide/47.5 ml of 50 mM acetate buffer, pH 5.0/50 μl of 30% H_2O_2). After 10 min the colored reaction develops, and the reaction is then stopped with H_2O; the color remains stable for several days.

Immunofluorescence Staining. Cells were grown on glass coverslips in Petri dishes, fixed with 3.7% *p*-formaldehyde (10–15 min), washed with P_i/NaCl, and permeabilized with ice-cold methanol (5 min at −20°C). Methanol was removed and replaced by ice-cold acetone (5 sec). Diluted antibody was added to each coverslip and incubated 30 min at 37°C. After three washes with P_i/NaCl, a 1:20 dilution of fluorescein-conjugated $F(ab')_2$ fragment of sheep anti-mouse Ig was added to each coverslip and incubated 10 min at 37°C. Coverslips were then washed three times with P_i/NaCl, washed once with distilled water, dried, and mounted in P_i/NaCl containing glycerol.

Cell-Free mRNA Translation. Human kidney was obtained by nephrectomy. Normal tissue was immediately excised and stored in liquid nitrogen. RNA was extracted by the guanidine·HCl method (14), modified by Alvino *et al.* (15). $Poly(A)^+$ RNA was isolated on an oligo(dT)-cellulose (16) column. $Poly(A)^+$ RNA was translated *in vitro* with a nuclease-treated cell-free rabbit reticulocyte lysate (17) in the presence of L-[^{35}S]methionine.

Immunoprecipitation. Protein A-Sepharose (50 μl of a 1:1 dilution in 40 mM Hepes buffer, pH 8.0) was coupled with anti-urokinase antiserum (1–2 μl) or to anti-urokinase mAbs (1.0–2.0 ml of culture medium of hybrids 52.C7, 65.F4, or 105.IF10) in the presence of 10% fetal calf serum for 1 hr at 4°C. Protein A-Sepharose-coupled antibodies were collected by centrifugation and washed twice with 40 mM Hepes (pH 8.0). To this pellet a sample of ^{35}S-labeled proteins synthesized in a cell-free rabbit reticulocyte lysate (17) was added together with 10% fetal calf serum/0.5% Nonidet P-40/Hepes, pH 8.0 (40 mM final concentration). Protease inhibitors (benzamidine, EDTA, aprotinin, and phenylmethylsulfonyl fluoride at 1 mM each) were present throughout the immunoprecipitation. Incubation was carried out for 2–4 hr at 4°C under continuous gentle agitation. Immunocomplexes were collected by centrifugation, washed six times with 40 mM Hepes/150 mM NaCl/0.5% Nonidet P-40, pH 8.0, and twice more with 40 mM Hepes (pH 8.0). Immunoprecipitated ^{35}S-labeled proteins were boiled 10 min in Laemmli buffer (18), and the supernatants were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (12.5% acrylamide). For ^{35}S detection, gels were fluorographed as described (19).

RESULTS

Characterization of Anti-Urokinase mAbs. mAbs to human urokinase were produced as described. In the experiments reported here, we used as the source of anti-urokinase mAbs either culture media of cloned hybrids or antibodies purified from ascites fluids from mice that had been injected with repeatedly cloned hybrid cells and were developing an ascites tumor.

The class specificity of the heavy chains of the anti-urokinase mAbs tested by double immunodiffusion, is reported in Table 1. Each mAb produced a single precipitation line with only one of the antisera.

In order to carry out a more refined characterization of the anti-urokinase mAbs, several of them were purified (from ascites fluids or from culture supernatants) either on protein A-Sepharose columns, or on urokinase-Sepharose columns. The type of column used depended both on the affinity constant of the antibody and on the class specificity of its heavy chain. For instance, antibody 65.F4 (IgG1) was routinely purified on protein A-Sepharose, whereas mAbs 52.C7 (IgM) and 59.II (IgG1) were purified onto a urokinase-Sepharose column.

Detection of Urokinase in Cultured Cells by Immunofluorescence. We used mAbs to detect urokinase-producing cells by immunofluorescence (Table 1). All mAbs are negative with human fibroblasts F13 that do not produce urokinase, although giving a positive response with the human kidney carcinoma line A1251 that produces urokinase (unpublished data). Fig. 1 shows immunofluorescence of A1251 cells with mAb 59-II, developed with fluorescein-conjugated goat anti-mouse $F(Ab')_2$ fragment. The specificity of the response was tested with other mAbs directed to different antigens and by the displacement of the immunofluorescence by preincubation of the antibody with authentic, purified urokinase (data not shown). Fig. 1 shows that the fluorescence was localized

Table 1. Species specificity of mAbs to human urokinase as studied by immunofluorescence staining of methanol-permeabilized cells

mAb	Ig class specificity*	Cell lines†			
		Human		Rat-2	
		A1251	F13	−TPA	+TPA
Nonimmune mouse serum		−	−	−	NT
59-II	γ1	++++	−	+	++
65-WL2	γ2a	+++	−	+	++
16-1E9	γ1	+++	NT	−	−
105.IF 10	γ1	++	NT	NT	NT
126‡	NT	++	NT	−	+
52C7	μ	NT	NT	NT	NT

*Tested by the double immunodiffusion procedure with class-specific rabbit anti-mouse antisera.

†A plus sign indicates the appearance of clearly recognizable immunofluorescence, disappearing when the antibody was preadsorbed with authentic highly purified human urokinase. NT, not tested.

‡Uncharacterized anti-urokinase mAb (serum-free hybridoma medium diluted 1:10 was used in this experiment).

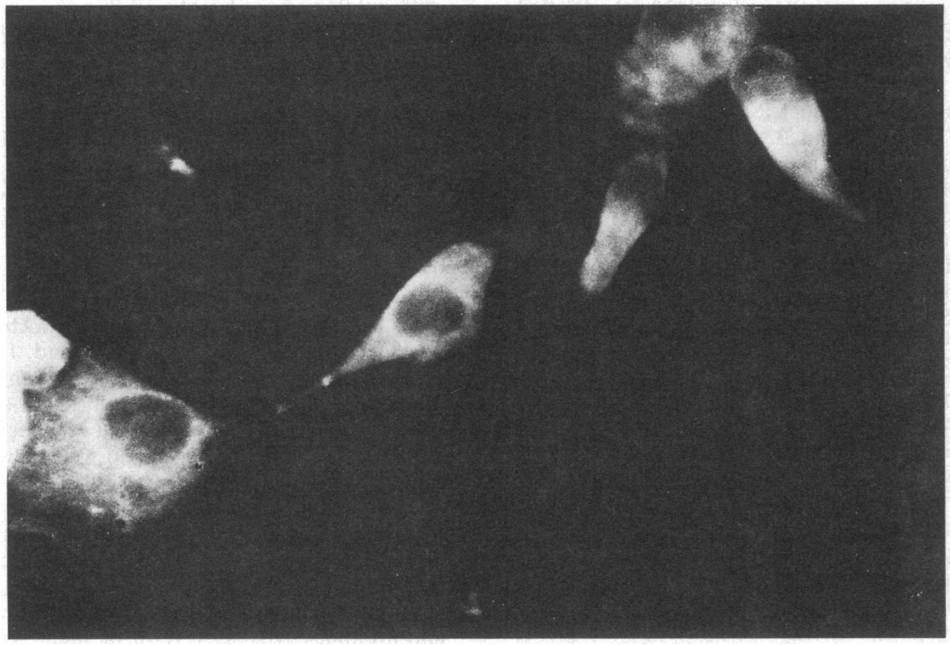

FIG. 1. Immunofluorescence of human kidney carcinoma A1251 cells permeabilized with methanol, treated with anti-urokinase mAb 59.II, and stained with fluorescein-conjugated anti-mouse IgG antiserum.

in the cytoplasm. Because urokinase is secreted by A1251 cells into the culture medium (unpublished data), it was of interest to investigate whether specific immunofluorescence could be visualized also in nonpermeabilized cells. No positive reaction, however, was observed in this case (data not shown).

Antiserum directed against a given antigen may crossreact with antigens of different species. mAbs, each being directed to single antigenic determinants, may discriminate between species. We challenged antibodies raised against purified human urokinase with human and rat plasminogen activator-producing cells and analyzed the reaction by immunofluorescence (Table 1).

Rat plasminogen activator-producing cells (Rat-2) were recognized by mAbs 59II and 65WL2, giving a weak, yet specific, fluorescence. Treatment of Rat-2 cells with 10 nM phorbol 12-tetradecanoate 13-acetate (TPA), which induces the synthesis of urokinase in several systems (3), increases the brightness of the staining. mAb 126, which did not react with rat-2 cells, did so if they were pretreated with TPA. mAb 16, on the other hand, did not react with Rat-2 cells, even when they were treated with TPA. It readily recognized, however, human urokinase-producing cells A1251.

Different mAbs Recognize Either the 30,000- or the 18,000-Dalton Chain of Urokinase. Upon prolonged incubation at room temperature, urokinase breaks down into 33,000- and 18,000-dalton peptides (7), which were separated by gel filtration. Purified peptides were run onto a 12.5% $NaDodSO_4$/polyacrylamide electrophoresis gel (with or without prior treatment with 2-mercaptoethanol), blotted onto nitrocellulose paper, and treated with mAb 52.C7, 65.F4, or 105.IF10. Binding of the antibody was demonstrated by a horseradish peroxidase-catalyzed color reaction. Both 52.C7 and 65.F4 mAbs gave the same result, and the data for 52.C7 are shown in Fig. 2. The antibody reacted with the purified B chain (lane 4) as well as with the 54,000-dalton urokinase (lane 5) but not with the purified A chain (lane 3). Treatment of either urokinase (lane 1) or purified B chain (lane 2) with 2-mercaptoethanol slightly increased the mobility of the immunoreactive chain. This shift depended on the loss of the 21-amino-acid minichain, which is disulfide-bonded to the B chain in the 33,000-dalton degradative form of urokinase (7). Thus, both mAbs 52.C7 and 65.F4 recognize the 30,000-dalton chain of urokinase.

mAb 105.IF10, on the other hand, recognized the purified A chain as well as the 54,000-dalton urokinase (Fig. 2, lanes 6 and 7). In conclusion, different mAbs recognize either the A or B chains of human urinary urokinase.

Cell-Free Translation of Urokinase mRNA. We have exploited the specificity of anti-urokinase antibodies for studying the production of urokinase in an mRNA-directed cell-free translation system, using a rabbit reticulocyte lysate and total human kidney poly(A)$^+$ RNA. The results obtained with mAbs 52.C7 and 65.F4, which recognize the B chain of urokinase, with mAbs 105.1F10, which is A chain-specific, and with rabbit anti-urokinase polyclonal antisera are compared in Fig. 3. In all cases (lanes 2, 3 and 5–9), a band of about 54,000-daltons was precipitated; in the immunoprecipitations with antiserum (slots 2, 3, 5, and 6), a second band was also precipitated (≈30,000 daltons). When immunoprecipitation was carried out in the presence of unlabeled purified urokinase, the 54,000-dalton band disappeared in all cases, while the 30,000-dalton band visible with the polyclonal antisera was not displaced (data not shown). Upon purification of monospecific anti-urokinase IgG from the polyclonal antiserum, this spurious immunoprecipitate disappeared (data not shown). Thus, the primary product of urokinase mRNA in human kidney is a single polypeptide of ≈54,000 daltons, which is precipitated by mAbs directed towards either the A or the B chain and, thus, represents the precursor, pro-urokinase, of the mature two-chain enzyme. The heterogeneity of the ≈54,000-dalton band in the immunoprecipitates with conventional antisera (compare slots 2, 3, 4, and 6 to slots 7–9) may be ascribed to the influence of the nonlabeled Ig heavy chains (used with polyclonal antisera) on the migration of the antigen.

From the data of Fig. 3 and similar experiments, we calculated that the percentage of ^{35}S in the immunoprecipitated product constituted about 0.05–0.1% of total ^{35}S incorporated *in vitro*. We carried out several experiments to exclude the possibility that these data are biased by factors like inefficient cell-free protein synthesis and immunoprecipitation

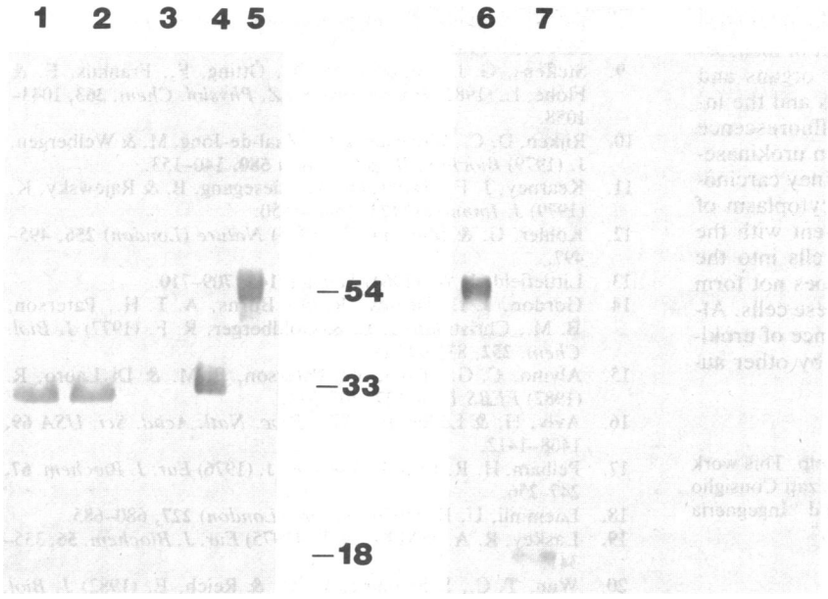

FIG. 2. Immunoblotting of human urinary urokinase with anti-urokinase mAbs 52.C7 (lanes 1–5) and 105.IF10 (lanes 6 and 7). Urokinase or its previously separated chains were run onto a 12.5% $NaDodSO_4$/polyacrylamide electrophoresis gel, the gel was blotted onto nitrocellulose, and the paper was treated with the mAbs and stained by ELISA: human 54,000-dalton urinary urokinase with (lanes 1 and 7) and without (lanes 5 and 6) prior reduction with 2-mercaptoethanol, the 33,000-dalton chain with (lane 2) and without (lane 4) reduction, and the 18,000 dalton chain unreduced (lane 3). Center numbers indicate the migration of 54,000-dalton urokinase and of its two chains B and A (mass is shown $\times 10^{-3}$).

or presence of translation inhibitors. Among these were urokinase mRNA size-fractionation, primer extension with synthetic oligonucleotides, and use of different sources of total or enriched poly(A)$^+$ RNA and of different reticulocyte lysates. We obtained no evidence that the urokinase mRNA may constitute >0.1% of total poly(A)$^+$ RNA either in adult or in embryonic human kidneys (data not shown). In addition, these results are in good agreement with the amount of urokinase synthesized by human kidney carcinoma cells in culture (unpublished data).

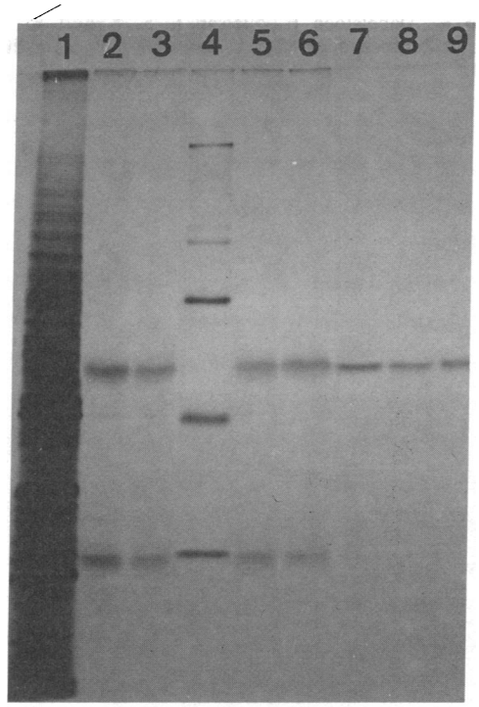

FIG. 3. Immunoprecipitation of cell-free products of human kidney total poly(A)$^+$ RNA, synthesized in a reticulocyte cell-free system, run through a 12.5% $NaDodSO_4$/polyacrylamide electrophoresis gel after reduction with 2-mercaptoethanol. Lanes: 1, 1.5 μl of total cell-free synthesized products; 2–9, 100 μl of total cell-free synthesized products immunoprecipitated with rabbit anti-urokinase antisera (lanes 2, 3, 5, and 6) or with mAb 52.C7 (lane 7), 65.F4 (lane 8), or 105.IF10 (lane 9); 4, size markers of 200,000, 69,000, 46,000, and 30,000 daltons.

DISCUSSION

Although the primary sequence of human urinary urokinase has been completely determined (7–9), the biosynthetic precursor, pro-urokinase, has not yet been identified. A single-chain 54,000-dalton polypeptide has been isolated from tumor cell lines (20, 21). However, larger forms also have been described (22, 23). We used mAbs to identify the product of urokinase mRNA in a cell-free translation system. The major reason for using mAbs lies in the existence of conflicting published data regarding the abundance of the urokinase mRNA and the size of its cell-free synthesized product.

It has been reported that human urokinase mRNA is ≈5,000 nucleotides long (i.e., a size consistent with a protein product of even 150,000 daltons) and that its cDNA copy cloned into *Escherichia coli* expresses a whole spectrum of proteins (ranging from 30,000 to 120,000 daltons) showing urokinase activity (24). These authors also report that this mRNA is very abundant in embryonic kidney cells, up to 10% of the total poly(A)$^+$ RNA. Another group has reported that proteins of ≈47,000 and 20,000 daltons and none of higher mass, can be immunoprecipitated by an anti-urokinase antiserum from the proteins synthesized in an *in vitro* cell-free system directed by embryonic or adult human kidney mRNA (25). The latter authors estimated the amount of urokinase mRNA to be ≈10% of the total kidney poly(A)$^+$ RNA. We have used mAbs to try to clarify these points. Our results (Fig. 3) show that the primary product of human urokinase mRNA is a single polypeptide of ≈54,000 daltons and that the second band of lower mass is likely to be a contaminant that can be eliminated also by using polyclonal antibodies purified on a urokinase-Sepharose column. In fact, the mAbs 52.C7 and 65.F4, which recognize the 30,000-dalton B chain of urokinase (see Fig. 2), and mAb 105-IF-10, which is directed towards the 18,000-dalton A chain, all immunoprecipitate one single 54,000-dalton *in vitro* synthesized protein. Secondly, urokinase mRNA is not an abundant mRNA, constituting at the utmost 0.1% of total poly(A)$^+$ RNA in adult (Fig. 3) and embryonic kidney cells (data not shown). The discrepant results obtained by other authors (24, 25) may be due to insufficiently specific antisera. In addition, because no other bands of higher molecular weight are precipitated by anti-urokinase antibodies, the higher molecular weight forms of urokinase (22, 23) do not seem to represent biosynthetic precursors of urokinase. It remains to be established whether or not pro-urokinase contains a signal peptide required for secretion.

In the course of this investigation, we obtained several anti-urokinase mAbs, which should prove useful in measuring the concentration of urokinase in different organs and tissues and for determining the site of synthesis and the intracellular distribution of urokinase. Immunofluorescence with mAb 59.II has been used, in fact, to screen urokinase-producing human cell lines. In A1251 human kidney carcinoma cells, fluorescence is only evident in the cytoplasm of permeabilized cells (Fig. 1). This is in agreement with the urokinase being rapidly secreted from these cells into the culture medium and indicates that urokinase does not form any stable relationship with the membrane of these cells. After this work was completed, immunofluorescence of urokinase-producing cells with mAbs was reported by other authors (26).

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