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SYNTHESIS IN VITRO OF UROKINASE-LIKE MATERIAL USING POLYA$^{(+)}$RNA FROM HUMAN KIDNEY AND FROM CULTURED HUMAN EMBRYONIC KIDNEY CELLS

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SUMMARY

Total RNA was extracted from human kidney or human embryonic kidney cells in culture, then passed through oligo-d(T) cellulose to obtain total polyA(+)RNAs. Messenger activity of the preparations was checked *in vitro* using the rabbit reticulocyte lysate system and peptides synthesized *in vitro* were separated on SDS-containing polyacrylamide gels. Identification of urokinase-like material was achieved by immuno-precipitation with antibodies raised against pure commercial urokinase and by radioimmunoassays where urokinase synthesized *in vitro* was competed out with unlabelled urokinase. The data show that both polyA(+)RNAs code for urokinase-like material and that the apparent Mw of the *in vitro* translations product is roughly 47 K daltons (in reducing conditions).

INTRODUCTION

Urokinase (E.C.3.4.99.26), an enzyme synthesized in human kidney (1,2) and released in urine (3,4), functions as an activator of plasminogen (3,5). Active plasmin resulting from the proteolytic cleavage of its precursor then exhibit a fibrinolytic activity. While the role of urokinase in human physiology is undoubtedly of great significance, the real interest in this enzyme resides in its therapeutic value for promoting dissolution of thrombi *in vivo* (for a review, see 6). Biochemical studies on urokinase demonstrated the existence of two major forms of the enzyme; one form has a molecular weight in the range of 47-54.000 daltons and the other in the range 31 to 33.000 daltons (7). It seems that the larger species is a precursor of the smaller form and that proteolytic, possibly autocatalytic cleavage, may convert the heavy species into smaller one. In addition, the fragments generated by cleavage seem to remain associated by disulfide bonds, yielding the 31 K

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active enzyme and a 18 K inactive fragment upon reducing. Both forms of urokinase possess activity and are used in therapy (8,9,10,11,12). Since urokinase appears to be, at this time, the sole plasminogen activator of human origin with therapeutic value, commercial production of the enzyme started a few years ago, essentially extracting urokinase from human urine or from spent medium of human kidney cells cultures. Both ways are expensive since the yield of urokinase recovered is relatively low (2). In this respect, gene splicing techniques could provide a way to increase the yield of urokinase by programming bacteria to synthesize the enzyme. A first step towards this goal consists of characterizing in vitro the messenger RNA coding for urokinase and its primary translation product. In this work, we describe experiments along these lines, using $polyA^{(+)}$ RNAs from human kidney and from human embryonic kidney cells in culture. The data indicate that both sources contain the messenger RNA coding for urokinase and that the primary translation product in vitro has an apparent molecular weight of roughly 47 K daltons in reducing conditions.

MATERIAL AND METHODS

a) Human kidneys were obtained, following nephrectomy, thanks to the courtesy of Dr. C. Schulman (Hopital Erasme, University of Brussels)
b) Human enbryonic kidney cells (HFK cells) were purchased from Flow Laboratories. Growth medium was Basal Medium Eagle with Earle's salts supplemented with 10% Fetal bovine serum and glutamine (2 mM). Growth to confluency was performed in Nunc flasks (175 cm2) in 5% CO_2 at 37°C.
c) Pure commercial urokinase was provided generously by Drs. Guzzi (Firma Lepetit, Milano) and Imai (Godo Shusei, Tokyo).
d) Antibodies against urokinase were raised in rabbits according to Stöffler and Wittmann (13).
e) Assay for urokinase activity was performed using the synthetic chromogenic substrate S-2444 (Kabi, Sweden), according to the manufacturer specifications.
f) Extraction of total RNAs.
1°) Human kidney

Following nephrectomy, organs were rinsed thoroughly with ice cold Tris-HCl buffer 100 mM, pH 8.3 containing 1 M sucrose and 0,15 M NaCl, then cut into small pieces and frozen immediately in liquid nitrogen.
To extract total RNAs, 7 grams of frozen kidney were ground in a mortar (kept cold with liquid nitrogen), then mixed with 10 volumes of TNE buffer (Tris HCl 100 mM, pH 8.3; NaCl 0.15 M, EDTA 0.01 M, SDS 1%) in an electric mixer. One volume of phenol-chloroform-isoamylic alcohol (90:9:1) was added to the resulting suspension which was then stirred for 20' at room

temperature and centrifuged for 20' at 10000 rpm (Sorvall HB4 rotor). The aqueous phase was reextracted four times with phenol. Total RNAs were then precipitated with two volumes of cold ethanol in the presence of 0.2 M NaCl and kept at -20°C.

2°) HFK cells

Cells deriving from 40 Nunc flasks at confluency were trypsinized, collected by low speed centrifugation, washed twice with phosphate-buffered saline (PBS) and resuspended in 60 ml TNE buffer. The suspension was then treated with phenol as described above and total RNA recovered by ethanol precipitation.

g) Isolation of polyA$^{(+)}$RNAs

Total RNAs (centrifuged 1 hour at 10.000 rpm and -10°C, and air dried) were dissolved in hybridization buffer (Tris-HCl 10 mM pH 7.5; EDTA 1 mM, NaCl 0.3 M and SDS 0.5%), heated for 1' at 90°C and mixed with oligo-d(T) cellulose type 7 (Collab, Research). The mixture was allowed to stand for 15' at 22°C then poured into a small column. The resin was washed with hybridization buffer until the eluate attained an OD_{260} of less than 0.01. PolyA(+)RNAs were eluted from the resin with elution^{260}buffer (hybridization buffer without NaCl). Appropriate fractions were pooled, precipitated with 2 volumes of cold ethanol in the presence of 0.37 M NaCl and stored at -20°C. When needed, polyA(+)RNAs were centrifuged (10.000 rpm, 20 min.) redissolved and reprecipitated with ethanol. Stock solutions of polyA(+) RNAs were kept at -80°C in bidistilled water at a concentration of 25 OD_{260}/ml.

h) Cell-free translation systems

PolyA(+)RNAs (1 µg) were translated in a cell-free system (25 µl) derived from nuclease-treated rabbit reticulocyte lysates (NEN) using ^{35}S -methionine as tracer (1 µCi/µl). The assay was performed according to the specifications of the manufacturer. Following incubation for 90' at 30°C, the reaction was terminated by the addition of pancreatic RNAse A to a final concentration of 10 µg/ml. Aliquots were withdrawn at various times for counting and the remaining of the samples was used for gel electrophoresis and for immunoassays.

i) Radioimmunoassays

Aliquots (10 µl) of cell-free translation systems were mixed with increasing amounts of commercial urokinase and allowed to react with antibodies against urokinase (10 µl of a normal serum). The system (200 µl) contained phosphate buffer 10 mM pH 8, NaCl 50 mM; 30 µg PMSF and 10 µg soybean trypsin inhibitor. Incubation was performed for 60' at 37°C and two hours at 25°C. Thereafter, 4 mgr protein A - sepharose (Pharmacia) conditionned in the same buffer was added to the mixture and allowed to react for 2 hours at 25°C with gentle stirring. The suspensions were washed five times with Tris-HCl 50 mM, pH 8, urea 8 M, SDS 1% and β-mercaptoethanol 1%, heated for 10' at 100°C and counted in a liquid scintillation counter (LKB). Alternatively, aliquots of translation systems were mixed with antiserum against urokinase as described above and antibody complexes were precipitated with goat anti-rabbit γ-globulins IgG (Nordic). In some instances, a pretreatment was achieved with non immune serum or with immune serum partially depleted from specific antiurokinase antibodies (by reaction with urokinase in liquid phase). Procedures for washing and analysis of the samples were as described above.

j) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

10 µl aliquots of translation systems were mixed in a one to one ratio with SDS buffer (Tris-CHl 100 mM pH 7, glycerol 20%, SDS 10%, β-mercaptoethanol 2%, bromphenolblue 1%), heated for 10' at 65°C and electrophoresed on a 15% polyacrylamide slab gel using the Laemmli system (14). Gels were stained with Coomassie blue, destained, dried and autoradiographed for 24 hrs to one week at -70°C.

RESULTS

Human fetal kidney cells produce urokinase.

The first experiment we performed was designed to confirm that HFK cells in culture secrete urokinase in the medium (2). Cells were grown to confluency in complete growth medium, and thereafter incubated for three days into medium without fetal bovine serum. The spent medium was cleared by centrifugation and assayed for urokinase activity using the chromogenic synthetic substrate S-2444. As seen in Figure 1, it is obvious that HFK cells release the plasminogen activator whereas no activity was detected with fresh medium.

Translation of polyA$^{(+)}$RNAs from human kidney and from HFK cells.

PolyA$^{(+)}$RNAs were extracted from human kidney and from HFK cells as described in Material and Methods. The yields were as follows: roughly 50 µg of polyA$^{(+)}$RNAs were recovered starting from 7 grams of frozen human

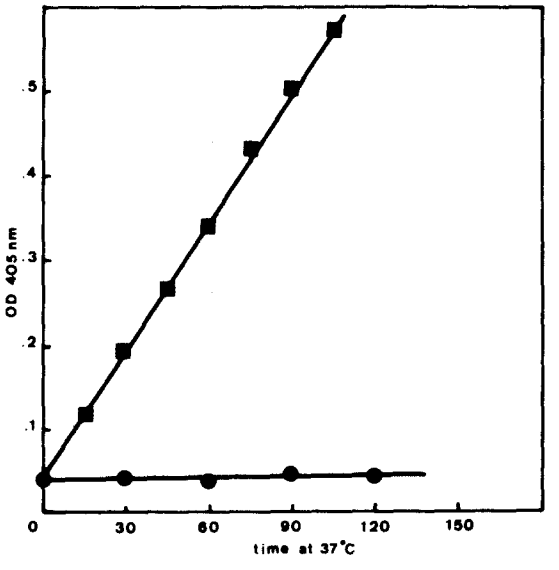

Fig. 1 - Assay for urokinase activity in spent medium using the chromogenic substrate S-2444 (Kabi, Sweden)

■ spent medium from HFK cells in culture, concentrated 25 times by ultrafiltration.
● fresh medium (concentrated 25 times)

The OD_{450} absorbption value measures the cleavage of the substrate by urokinase (release of p-nitroaniline).

kidney and 500 µg of polyA $^{(+)}$RNAs were obtained starting from 40 Nunc flasks of HFK cells at confluency (about 7 ml of packed cells). In the reticulocyte lysate system, polyA$^{(+)}$RNAs from both sources stimulated the incorporation of radiolabelled amino acid into protein 5-15 fold over the amount incorporated in the absence of added messenger RNA.

As seen in Figure 2, the activity of polyA$^{(+)}$RNAs from kidney and from HFK cells was equivalent to the activity of control mRNA (globin) for a similar input (1 OD_{260}/ml). Sodium dodecyl sulfate polyacrylamide gel analysis of the translation products revealed the presence of several polypeptides of molecular weight ranging from 80 to 15 kilodaltons (Fig 3).

Dosage of the urokinase specific polyA$^{(+)}$RNA by radioimmunoassay.

In order to quantitate the amount of polyA$^{(+)}$RNA specific for urokinase among the total polyA$^{(+)}$RNAs, we performed the following assay. Putative

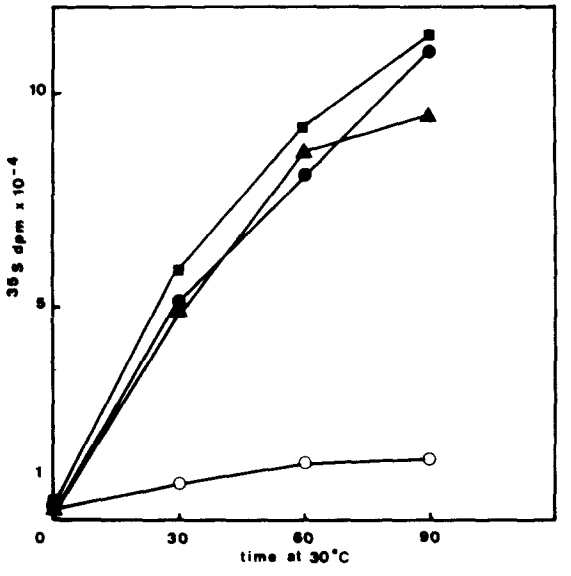

Fig. 2 - Incorporation of ^{35}S -methionine in rabbit reticulocyte lysate systems directed by polyA $^{(+)}$RNAs
PolyA(+)RNAs (1 µg) were translated in vitro using 25 µl of rabbit reticulocyte lysate system (NEN) with ^{35}S -methionine as tracer (1 µCi/µl)

▲ globin messenger RNA
■ human kidney cells polyA$^{(+)}$RNAs
● human kidney polyA(+)RNA
○ no added RNA

Mixtures were incubated at 30°C. Aliquots (2 µl) were collected every 30 minutes, treated with RNAse A, TCA precipitated, boiled, filtered on millipore and counted.

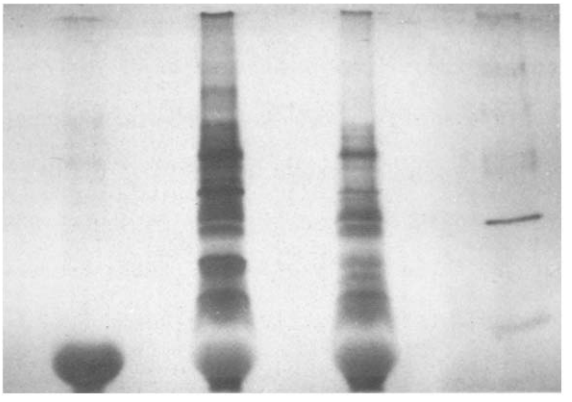

Fig. 3 - Cell-free translation products of polyA$^{(+)}$RNAs from human kidney and HFK cells
PolyA(+)RNAs from (A) globin mRNA, (B) HFK cells and (C) human kidney were translated in a rabbit reticulocyte lysate system (1 µg RNA in 25 µl reaction mixture). Products were labelled with ^{35}S -methionine (1 µCi/µl). 10 µl aliquots were submitted to gel electrophoresis in reducing conditions and autoradiographed as described under "Methods". Molecular weight standards consisted of a mixture of ^{14}C methylated proteins (Amersham): myosine (Mw: 200.000); phosphorylase - b (Mw: 100.000 and 92.500); bovine serum albumine (Mw: 69.000); ovalbumin (Mw: 46.000); carbonic anhydrase (Mw: 30.000) and lysozyme (Mw: 14.300).

urokinase synthesized in vitro was competed out with pure urokinase in the reaction with antibodies raised against the enzyme. The experiment consisted of incubating aliquots of translation systems with various amounts of urokinase in the presence of antibodies and then to recover the complexes with protein A-sepharose. As seen in Table 1, both kidney and HFK cells polyA$^{(+)}$RNAs code for polypeptides having antigenic determinants accessible to urokinase antiserum. In addition, it is clear that the putative urokinase synthesized in vitro is competed out of the reaction by pure commercial urokinase. On the basis of the input of label in the reaction, we can compete out about 10% of the labelled polypeptides (see Table 1). This value may reflect the actual proportion of polyA$^{(+)}$RNA specific for urokinase.

Molecular weight of the urokinase-like material synthesized in vitro. Aliquots of translation systems precipitated with specific urokinase antiserum were analysed on SDS-polyacrylamide gels in reducing conditions. The major products identified by autoradiography have apparent molecular

TABLE 1

Indirect quantitation of polyA$^{(+)}$RNA specific for urokinase by radioimmunoassay.

Source of polyA$^{(+)}$RNAs	mature urokinase in the assay (µg)	immunoprecipitable material (dpm)
HFK cells	0	9568
Input of translation	2.5	7604
products	5	4543
^{35}S -methionine	10	4154
55.765 dpm	20	2958
Human kidney	0	9622
Input of translation	2.5	6311
products	5	5772
^{35}S -methionine	10	5519
47.235 dpm	20	4053
Control m RNA (globin)	0	2964
Input of translation	2.5	2141
products	5	2073
^{35}S -methionine	10	2413
27.507 dpm	20	1801

The input of translation products is estimated on the basis of TCA precipitable material. The conditions for the assay are described under "Methods".

weights of about 47 and 20 K daltons (Figure 4 A and C). When samples were pretreated with non immune antiserum, immunoprecipitates showed only the 47 K species on gel (Figure 4 B and D). In addition, the use of immune serum preadorbed with urokinase (liquid phase) lead to the partial disappearance of the 47 K product (data not shown). When gels were exposed for longer periods, additional bands were detected on the autoradiographs. These products may be contaminants coprecipitating with urokinase in the immuno-assays or could be minor components resulting from autocatalytic cleavage of urokinase-like material. The fact that experiments were carried out in reducing conditions could also account for the detection of additional fragments on the gels. In conclusion, we propose that the

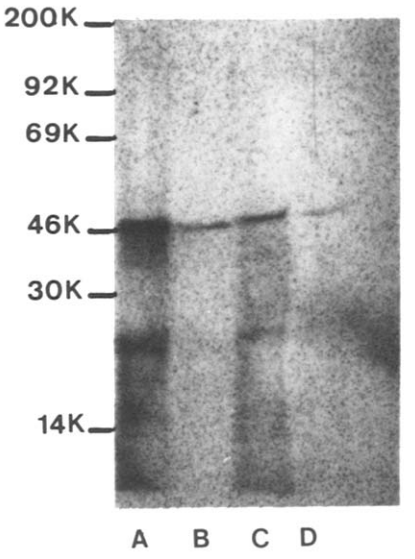

Fig. 4 - Immunoprecipitation of cell-free translation products by urokinase antiserum
Aliquots of translation products directed by polyA$^{(+)}$RNAs from human kidney and HFK cells were subjected to double antibody precipitation as described under "Methods" and analysed on SDS-polyacrylamide gels in reducing conditions. Autoradiographs of the gels are shown

immunoprecipitation with urokinase antiserum	A) HFK cells polyA$^{(+)}$RNAs C) kidney polyA$^{(+)}$RNAs
immunoprecipitation with urokinase antiserum following pretreatment with non-immune serum	B) HFK cells polyA$^{(+)}$RNAs D) kidney polyA$^{(+)}$RNAs

47 K daltons material represents the major translation product in vitro in reducing conditions.

DISCUSSION

Our results indicate that translation products from both human kidney and human fetal kidney cells polyA$^{(+)}$RNAs contain proteins with antigenic determinants similar to urokinase.

The major translation product reacting specifically with urokinase antiserum has a molecular weight of about 47000 daltons in reducing conditions. Although the significance of less prominent immunoprecipitable material is not clear, it may reflect either post-translational modification of a single product (autocatalytic cleavage, etc...) or translation artifacts

such as premature termination. The data presented here suggest that roughly 10% of total polyA$^{(+)}$RNAs from both sources studied code for urokinase-like material.

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