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EXPRESSION IN ESCHERICHIA COLI OF UROKINASE ANTIGENIC DETERMINANTS

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SUMMARY

Hybrid genes were constructed between the β-lactamase gene of plasmid pBR322 and enzymatically synthesized DNA sequences coding for urokinase-like material. The recombinant plasmids, pULB1003 and pULB1010, contain DNA inserts of 220 and 535 base pairs respectively, which appear to be in the correct reading frame. The hybrid genes are expressed in Escherichia coli and their products are specifically immunoprecipitated with antiserum to urokinase. In addition injection into a rabbit of a crude extract from the clone pULB1010 leads to the production of specific antibodies against urokinase.

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). In this respect, urokinase has a therapeutic value for promoting dissolution of thrombi in vivo and has been produced therefore commercially either from human urine or from spent medium of human kidney cells in culture (6). We reported previously (7) that total polyA$^{(+)}$ RNAs from human kidney and from human embryonic kidney cells in culture contain the messenger RNA specific for urokinase. We identified its translation product in vitro by immunoprecipitation with antibodies raised against urokinase. In this report, we describe the construction and characterization of recombinant DNA molecules that contain part of the urokinase coding sequences inserted at the PstI site of plasmid pBR322. Two hybrid plasmids, pULB1003 and pULB1010, carrying DNA inserts of 220 and 535 base pairs respectively, express polypeptides, in whole bacteria and in minicells, which are recognized specifically by antibodies raised against urokinase. In addition, we detected the production of specific

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antibodies against urokinase in a rabbit injected with a crude extract from clone pULB1010.

MATERIALS AND METHODS

Construction of hybrid DNA molecules and transformation

Total polyA$^{(+)}$ RNAs from human kidney and from human kidney cells in culture were prepared as described previously (7). These RNAs preparations served as template for reverse transcriptase (from avian myoblastosis virus, kindly provided by Dr. J. Beard) to prepare the complementary DNA which was then converted to the double stranded DNA (cDNAds) using DNA polymerase (8). The cDNAds was digested with S1 nuclease and deoxy(C) extensions (approximately 15 to 30 residues) were added by terminal transferase to 3' ends of the molecules (9). Plasmid pBR322 DNA was linearised with the enzyme PstI and tailed with deoxy(G) residues as described above (approximately 15-30 residues). Mixtures of equimolar amounts of cDNAds fractions and pBR322 DNA were annealed and used to transform Escherichia coli strains DP50 (λcI857) or MM294 (λcI857) as described by Villa-Komaroff (10). Transformed cells, selected on rich medium plates containing 15 µg/ml tetracycline, were screened for the ampicillin sensitive phenotype. Tet^R Amp^S transformants were screened for expression of urokinase antigenic determinants using the technique of Broome and Gilbert (13).

Antibodies

Antibodies against pure urokinase (Lepetit, Milano), against pure β-lactamase (a gift of Dr. R. Ambler) and against crude extracts from various transformants were raised in rabbits according to (11). Whenever necessary, IgG fractions were purified by chromatography on protein A-Sepharose (12) and eventually iodinated (^{125}I) as described in (13).

Analysis of plasmid-encoded proteins in minicells and immunoprecipitation

The minicells producing strain Escherichia coli DS410 were transformed with the plasmids giving a positive signal in the radioimmunodetection assay. Minicells were purified by zone sedimentation through 10-30 % sucrose gradients (14) and resuspended in methionine-free M9-salt medium. After 30 minutes preincubation at 37°C, [^{35}S]-methionine (Amersham, 500 Ci/mmole ; 50 µCi for 1 A_{260} of minicells) was added and incubation continued for 30 minutes at 37°C. Minicells were incubated for 10' at 37°C in the presence of lysozyme (2 mg/ml) in TES buffer (TrisHCl 100 mM pH8, EDTA 100 mM, sucrose 25%), at 4°C for 20' and exposed to four successive freezing and thawing cycles. Minicells were then sonicated for 30" (6 times) at 4°C. Lysates were made 0.5 % final in Triton X100, incubated for 15' at 4°C and spun for 10' at 10,000 rpm in the cold. Extracts were analysed by electrophoresis on 15 % polyacrylamide gels containing sodium dodecylsulfate (15). The labelled proteins were identified by autoradiography of the dried gels.

Aliquots of minicells extracts were mixed with 5 µl of anti-urokinase serum and incubated for 30 minutes at 37°C. Thereafter, 150 µl of sheep anti-rabbit serum was added to the mixture which was then further incubated for 16 hours at 4°C. The immunoprecipitates were washed several times with Tris HCl buffer, 10 mM pH8, containing 500 mM KCl and 1% Triton X100, then with Tris HCl 10 mM pH8. The final pellet was dissolved in SDS sample buffer (Tris HCl 50 mM pH8, 1% SDS ; 6 M urea, 5% glycerol and 1% β-mercapto-ethanol), heated for 10 minutes at 100°C and either counted in a liquid scintillation counter or applied onto SDS polyacrylamide gels. Labelled proteins were identified by autoradiography of the dried gels. Immunoprecipitation with β-lactamase antiserum was performed using a similar procedure. All sera used in these experiments were pretreated with a crude extract of strain MM294 (λcI857) to minimize non specific adsorptions.

Solid phase radioimmunoassay of anti-urokinase antibodies

Solid phase RIAs were performed on polyvinylchloride plates (16). Microtiter plate wells were coated with 25 µl of urokinase solution (8 µg/ml) by overnight incubation of 4°C. After removal of the solution, the wells were washed with phosphate saline buffer pH8 (PBS), filled with 3% bovine serum albumin in PBS and incubated at 37°C for 8 hours. After washing with PBS, 25µl of the sera to be tested were pipetted into the wells and the plates incubated at 37°C for 4 to 6 hours. The solutions were then removed and the wells were washed with PBS. The wells were then filled with 25 µl of a solution of [^{125}I]-labelled protein A (25 ng and 300.000 cpm per well, in PBS containing 0.1% bovine serum albumin). The multiwell plates were incubated overnight at 4°C, washed extensively with PBS, dried, cut and counted in a gamma radiation counter.

Restriction enzyme digestions

Restriction enzymes were obtained from New England Biolabs. Restriction fragments were sized on 1% agarose gels (Tris Acetate 40 mM pH 7.6, Sodium Acetate 5 mM, EDTA 1 mM), or on 5% polyacrylamide gels (Tris borate 90 mM pH 8.2, EDTA 2.5 mM). Bands were visualized by coloration with 0.5 µg/ml of ethidium bromide. Whenever necessary, gel bands were recovered by electroelution and ethanol precipitation.

RESULTS

Synthesis and cloning of cDNAds from human kidney and human kidney cells

Approximately 1 µg of cDNAds was synthesized from about 7 µg of total imput polyA$^{(+)}$RNAs. Following S1 nuclease treatment and tailing with [^{32}P]-labelled d(C) residues, the material was annealed to d(G) tailed pBR322 plasmid DNA and used to transform Escherichia coli strains DP50(λcI857) or MM294(λcI857). A total of 832 transformants originating from the cloning of cDNAds molecules were obtained (439 from the kidney cell source and 393 from the kidney source). Among these, 50% showed the ampicillin sensitive phenotype.

Screening of the Amp^S transformants for the expression of urokinase antigenic determinants.

The Amp^S transformants were screened for the expression of urokinase antigenic determinants using the procedure developped by Broome and Gilbert (13). In short, transformants were replica-plated on rich medium dishes containing tetracycline (15 µg/ml), grown overnight at 32°C, exposed at 42°C for 2 hours to allow lysis by the thermoinducible λ phage and then treated with chloroform vapors for 10 minutes. Polyvinyl disks coated with purified IgG molecules derived from anti-urokinase antibodies were applied onto the lysed colonies for 4 to 5 hours at 4°C, washed as described by Broome and Gilbert (13) and

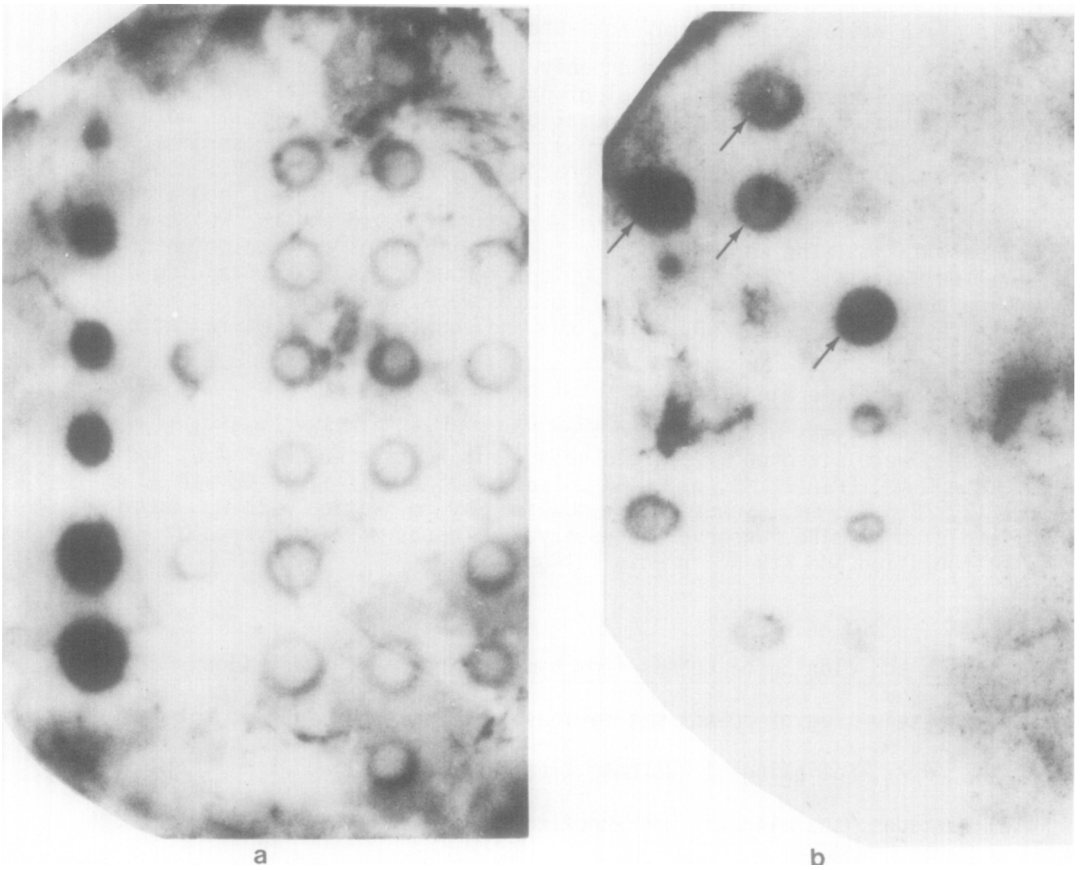

Figure 1 - Solid phase radioimmunodetection of clones expressing urokinase antigenic determinants. Antigen released from host cells lysed in situ was immobilized on polyvinyl disks coated with anti-urokinase IgGs and subsequently labelled with [^{125}I]-labelled anti-urokinase IgGs (13). Disks were then autoradiographed. a) urokinase standards : 30 pg, 60 pg, 120 pg, 240 pg, 480 pg and 960 pg. b) Tet^{R} Amp^{S} clones. Arrows point to positive signals.

exposed overnight to a solution containing [^{125}I]-labelled anti-urokinase IgGs. After extensive washing and rinsing, disks were air dried and autoradiographed for several days (4-10 days). Figure 1a shows the detection of control urokinase preparations using the method described above. It can be seen that a positive signal can be observed with as little as 30 pg urokinase (1 µl drops adsorbed on rich medium plates before impregnation with the polyvinyl disk). Figure 1b shows the signal obtained with several transformants; among a majority of negative colonies, several clones gave a positive signal

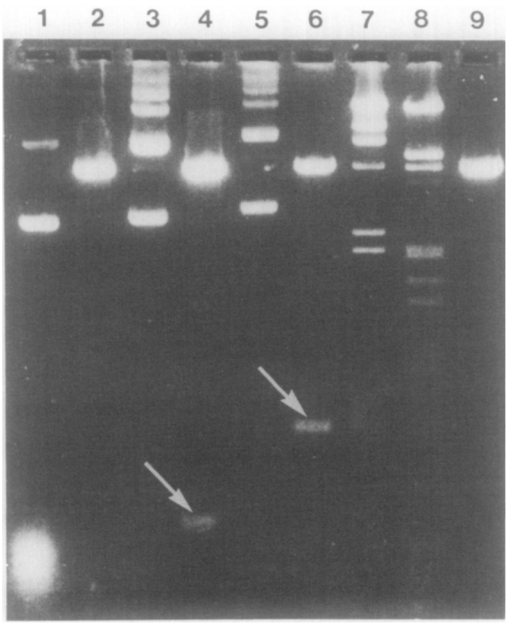

Figure 2 - Sizing of cDNAds inserts by agarose gel electrophoresis.
Electrophoresis on 1% agarose gels was for 6 hours at 100 mA.
1. intact pBR322 DNA
2 & 9. pBR322 DNA cut with PstI
3. intact pULB1003 DNA
4. pULB1003 DNA cut with PstI
5. intact pULB1010 DNA
6. pULB1010 DNA cut with PstI
7. HindIII cut λDNA, molecular size standards
8. HindIII and EcoRI cut λDNA, molecular size standards.
Arrows point to the DNA inserts.

in the assay. Two transformants, giving the strongest signals, were recovered for further analysis ; each of them carries a recombinant plasmid called pULB1003 and pULB1010.

Size determination of the DNA inserts in the transformants carrying plasmids pULB1003 and pULB1010.

Plasmids contained in the two positive transformants were isolated by CsCl gradient centrifugation and analysed on 1% agarose gels in the intact and PstI-digested forms. Figure 2 shows the comparison of DNAs from pBR322, pULB1003 and pULB1010. It can be seen that pULB1003 contains a DNA insert of about 220 base pairs and pULB1010 an insert of about 535 base pairs.

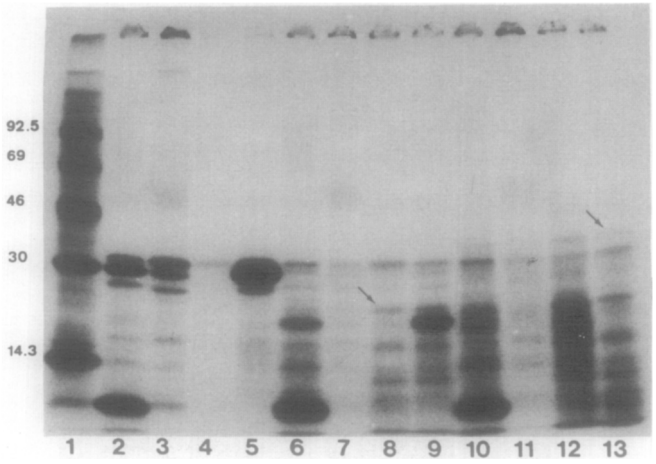

Figure 3 - Fluorogram of sodium dodecyl sulfate polyacrylamide gel showing the labelled polypeptides synthesized in plasmid containing minicells.
- Lane 1 : Mw standards
- Minicells containing plasmid pBR322
  Lane 2 : total extract
  Lane 4 : immunoprecipitation with anti-urokinase serum
  Lane 5 : immunoprecipitation with anti-β-lactamase serum
- Minicells containing plasmid pULB1003
  Lane 6 : total extract
  Lane 8 : immunoprecipitation with anti-urokinase serum
  Lane 9 : immunoprecipitation with anti-β-lactamase serum
- Minicells containing plasmid pULB1010
  Lane 10 : total extract
  Lane 12 : immunoprecipitation with anti-urokinase serum
  Lane 13 : immunoprecipitation with anti-β-lactamase serum

Arrows point to fused polypeptides.
(Lane 3, 7 and 11 show polypeptides present in the minicells incubation medium. They show essentially the same patterns as the ones presented in lanes 2, 6 and 10).

Expression of polypeptides encoded for by pULB1003 and pULB1010 DNAs.

In order to confirm the expression in the two transformants of polypeptides specifically recognized by anti-urokinase antiserum, we transformed a minicell producing strain (DS410) with the DNA from plasmids pULB1003, pULB1010 and pBR322 as control. The plasmids were reisolated from individual transformants to verify by PstI digestion that the DNA inserts were unchanged. Minicells containing each of the three plasmids were prepared and labelled with $[^{35}S]$-methionine as described in Materials and Methods. Aliquots of crude lysates were removed to determine 1) the total amount of label incorporated into protein (TCA precipitable material) and 2) the radioactivity

precipitable with rabbit antisera raised against urokinase or β-lactamase. In minicells containing pULB1010, 19.2% of the proteins coded for by the plasmid are immunoprecipitated by urokinase antiserum. This represents a 10 fold increase with respect to the control value (pBR322). Minicells containing pULB1003 also appear to synthesize urokinase-like material although to a lesser extent than those carrying pULB1010 (4 fold above the pBR322 background). In addition, the three minicells preparations clearly synthesize polypeptides recognized by β-lactamase antiserum (24% for pBR322 ; 12% for pULB1003 and pULB1010). Immunoprecipitates were then analyzed on 15% SDS polyacrylamide gels and polypeptides were detected by autoradiography. The most prominent proteins coded for by pBR322 (Fig 3, lane 2) correspond to the precursor and mature forms of β-lactamase (28, 29 and 32,000 daltons, see (17,18)). In this control experiment, urokinase antibodies do not precipitate significant amount of proteins since no discrete material can be observed on the gel (lane 4). On the contrary and as expected (lane 5), β-lactamase antiserum precipitates the three β-lactamase species found in the total extract (lane 2). These polypeptides are missing or at least greatly reduced in minicells containing the recombinant plasmids (Fig 3, lane 6 and 10). This is expected since the DNA inserts were cloned at the PstI site of the β-lactamase gene (Amp). Instead of intact β-lactamase, one observes several polypeptides of various sizes. Some of these molecules immunoprecipitate either with urokinase antibodies (lane 8 and 12) or with β-lactamase antibodies (lane 9 and 13). Minicells carrying pULB1003 contain a polypeptide which is recognized by both urokinase and β-lactamase antisera (lane 8). It has a molecular weight of roughly 22 K daltons, that is about 3 K daltons heavier than the main β-lactamase fragment (182 amino acids ; 19 K daltons) found in lane 9. This polypeptide corresponds probably to a fused product. In addition, one observes the presence of several short polypeptides precipitating only with anti β-lactamase which may be incomplete molecules (lane 9). Lane 8 and 9 also contain various common polypeptides which are probably degraded forms of the fused product.

Minicells carrying pULB1010 clearly synthesize a product (lane 10, 12, 13) whose molecular weight of 36 K daltons is in agreement with a fusion of the 182 first amino acids of β-lactamase (up to the PstI cleavage site) and of the material coded for by the 535 base pairs DNA insert (roughly 17,000 daltons). This fused product appears to be cleaved spontaneously since as seen in lane 12, a 17 K daltons species immunoprecipitate only with anti-urokinase. In addition to these products, one detects also β-lactamase fragments and degradation products of the fused molecules. Altogether the data shown in figure 3 support the conclusion that urokinase antigenic determinants are expressed in pULB1003 and pULB1010 and that they are part of a fused translation product containing the N-terminal portion of β-lactamase. It should be noted that the amount of fused protein from pULB1003 and pULB1010 is not as great as the amount of β-lactamase from pBR322. This might reflect the fact that fused proteins are not synthesized as efficiently as the normal product or that fused proteins are quickly degraded in the minicells. This last possibility is supported by the detection of small polypeptides immunoprecipitable by both urokinase and β-lactamase antisera.

Production of antibodies against urokinase in rabbits injected with crude extracts of clone pULB1010.

On the basis of the data presented above, one expects that the urokinase-like polypeptides synthesized in clones pULB1003 and pULB1010 would be immunogenic and thus stimulate the production in rabbits of antibodies which would specifically recognize urokinase. In order to check this possibility, we grew one liter cultures of bacteria carrying pULB1003, pULB1010 and pBR322 as control. These bacteria were broken in the French Press in phosphate saline buffer containing 1% nonidet P40, centrifuged to remove membranes and sterilized by millipore filtration. The extracts were then injected into rabbits as described in Materials and Methods. Sera (or partially purified γ-globulins) were used in the solid phase radio-immunoassay described in Materials and Methods. We found that the rabbit injected with the pULB1010 extract develop antibodies

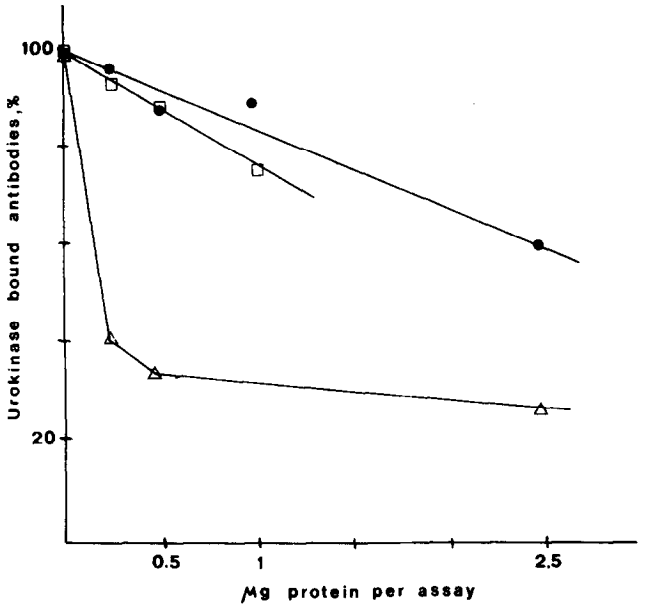

Figure 4 - Specificity for urokinase of antibodies raised in rabbits injected with crude extracts of clone pULB1010. Solid phase radio-immunoassays were performed as described in Materials and Methods. Serial dilutions of urokinase were incubated with sera derived from rabbits injected with crude extracts from pBR322 and pULB1010 containing bacteria. The mixtures were then allowed to react with polyvinyl-bound urokinase and specific complexes were detected with [^{125}I]-labelled protein A.
● pBR322 bacterial extract + urokinase at various concentrations
△ pULB1010 bacterial extract + urokinase at various concentrations
□ pULB1010 bacterial extract + bovine serum albumin at various concentrations.
100% on the ordinate corresponds to 2,436 cpm ^{125}I-labelled protein A for the pULB1010 bacterial extract. In the case of the pBR322 bacterial extract, 100% corresponds to 373 cpm.

which recognize the urokinase bound onto the multiwell plate. To find out whether this recognition is specific, we performed competition experiments where the sera to be tested were preincubated with various amounts of urokinase before being allowed to react with the bound urokinase. As seen in Fig.4, it is clear that the antibodies raised in the rabbit injected with the pULB1010 extract are indeed specific for urokinase. These data thus confirm that the plasmid pULB1010 codes for a fraction of the urokinase molecule and that the DNA insert is in the correct reading frame. Rabbits injected with crude extract from transformant pULB1003 did not develop antibodies against urokinase. This might be due to the small size of the polypeptide coded for by the 220 base

pairs DNA insert (insufficient immunogenicity). Alternatively, it is possible also that the level of antibodies produced is too low to be detected in the assay.

CONCLUSION

The PstI site in the β-lactamase gene of plasmid pBR322 was used for insertion of urokinase DNA seqences. Two recombinant plasmids, pULB1003 and pULB1010, were able to direct in bacteria the synthesis of urokinase-like polypeptides. The immunological characterization of these peptides employed two types of assays : in situ colony radioimmunoassay and synthesis in plasmid containing minicells. In both assays, extracts from the pULB1003 and pULB1010 clones reacted with anti-urokinase antibodies. In addition, urokinase-like polypeptides synthesized in bacteria carrying the pULB1010 plasmid were shown to be immunogenic in rabbits, leading to the production of antibodies specific for urokinase. We propose therefore that the cDNA inserts of clones pULB1003 and pULB1010 are in the correct reading frame and code for part of the urokinase gene.

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