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Biochemistry

Expression in *Escherichia coli* of biologically active enzyme by a DNA sequence coding for the human plasminogen activator urokinase

(recombinant DNA/fibrinolysis/human enzyme)

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ABSTRACT **We have isolated clones of *Escherichia coli* strain K-12 that contain a hybrid pBR322 plasmid having a 4.2-kilobase insert of a DNA transcript of the mRNA of human plasminogen activator, urokinase. The bacterially produced enzyme has properties similar to those of urokinase from human fetal kidney cells. Both enzymes occur in discrete forms ranging from 32,000 to 150,000 daltons in size. They react with antibody to purified urokinase from human kidney cells, bind to a benzamidine-Sepharose column, and induce plasminogen-dependent lysis of a fibrin clot.**

For the dissolution of a blood clot, it is necessary to activate plasminogen to form the protease plasmin which then degrades the fibrin network of the clot. A plasminogen activator called "urokinase" was first observed in human urine in 1951 by Williams (1). Urokinase-like activity has been found in cultures of human kidney cells (2), and the material was subsequently shown to be immunologically indistinguishable from urokinase derived from urine. In urine, as well as in tissue culture, urokinases of two sizes are found; they are called "type S_2" and "type S_1" and have molecular weights of 54,700 and 31,400, respectively (3, 4).

Human urokinase can be used to treat acute thromboembolic events such as venous and arterial thrombosis, pulmonary embolism, intracardiac thrombosis, and systemic embolism. However, the high cost of isolation of urokinase from either tissue culture cells or urine limits the use of this enzyme as a therapeutic agent. If urokinase could be obtained from microorganisms by recombinant DNA technology, one might have a more economical method of production. This report describes the construction of hybrid bacterial plasmids that produce human urokinase in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial Strain. *E. coli* K-12 strain χ1776 (5) (F^- *tonA53, dapD8, minA1, minB2, supE42,* 40 *(gal-uvrB)* λ^-, *rfb-2, nalA25, oms-2, thyA57, metC65, oms-1,* 29 *(bioH-asd), cycB2, cycA1, hsdR*) was provided by R. Curtiss III.

DNA and Enzymes. Plasmid pBR322 was isolated as described by Ratzkin and Carbon (6). Reverse transcriptase was obtained from Life Sciences (St. Petersburg, FL). *E. coli* DNA polymerase I (the large fragment) and various restriction enzymes were purchased from New England BioLabs. Terminal transferase was obtained from P-L Biochemicals and nuclease S1 was purchased from Miles. Digestions with restriction enzymes were carried out under conditions recommended by the supplier.

Antibody Against Urokinase. Anti-urokinase antibody synthesis was induced in New Zealand White rabbits by injection of purified urokinase (0.8 mg, type S_1) emulsified in Freund's adjuvant. After three more injections of antigen over a period of 2.5 months, the rabbits were bled. The antibody was purified by ammonium sulfate precipitation, followed by DEAE-cellulose chromatography and urokinase affinity chromatography (7). This antibody was checked for purity by electrophoresis (8) and for activity by the Ouchterlony test (9).

Isolation of mRNA from Human Fetal Kidney Cells. Human fetal kidney cells were grown to confluency in tissue culture (10) and were maintained in a protein hydrolysate medium for an additional 7-day period before harvest. Total RNA was isolated by the guanidine thiocyanate method (11). Poly(A)-containing mRNA was isolated from the total RNA by affinity chromatography on a poly(U)-Sephadex column as described (12). Total yield was 1.1 mg from 39 mg of total RNA. The RNA was enriched for urokinase mRNA by centrifugation in a 10–30% linear sucrose density gradient in 10 mM Tris·HCl, pH 7.4/20 mM NaCl, 0.5 mM EDTA/0.4% $NaDodSO_4$. Centrifugation was carried out in a SW 27 rotor for 20 hr at 25,000 rpm and 20°C in a Beckman L5-65 centrifuge. The RNA with sedimentation coefficient >28 S was pooled and precipitated by ethanol. From 1 mg of poly(A)-containing mRNA, about 70 μg of RNA with sedimentation coefficient >28 S was obtained. It was this fraction that possessed the highest messenger activity in urokinase biosynthesis.

Demonstration of the Presence of Urokinase mRNA by Cell-Free Protein Synthesis Systems. The cell-free synthesis systems from rabbit reticulocytes (13) and wheat germ (14) were used to demonstrate the presence of urokinase mRNA in mRNA preparations. Immunoprecipitation of the radioactive protein was done as described by Rhoads *et al.* (15). Characterization of the urokinase mRNA will be described elsewhere.

cDNA and Recombinant DNA Syntheses. Reverse transcriptase (16) was used to synthesize single-stranded cDNA from the mRNA with sedimentation coefficient >28 S. This DNA, after alkali treatment, was made double-stranded by use

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of DNA polymerase I (17). The double-stranded cDNA was then treated with nuclease S1 for 3 hr at 15°C in 30 mM sodium acetate, pH 4.6/1 mM $ZnSO_4$/250 mM NaCl containing 100 μg of *E. coli* tRNA per ml. The DNA was then centrifuged in a linear sucrose density gradient (15–30% in 10 mM NaCl/10 mM Tris, pH 8.0/1 mM EDTA) in a SW 40 rotor for 16 hr at 40,000 rpm. The fractions containing DNA larger than 2000 base pairs were pooled, and DNA was precipitated with ethanol. Poly(dC) tracts were attached to this cDNA (18). On the average, 70 dCMP residues were added to each 3′-OH end. The circular pBR322 DNA was digested by *Pst* I and purified, and poly(dG) tracts were attached to the linear DNA. An average of 24 dGMP residues were added to each 3′-OH end. The double-stranded cDNA that had been tailed with poly(dC) (0.15 nmol) was hybridized to an equimolar amount of the above pBR322 DNA in 100 μl of 0.1 M NaCl/10 mM Tris, pH 8.0/1 mM EDTA. The mixture was heated to 65°C for 3 min and left at 42°C for 16 hr and was then used to transform *E. coli* cells.

Transformation of *E. coli*. The procedure of Curtiss *et al.* (19) was used to transform strain χ1776 with the hybridized DNA mixture described above. The transformed cells were incubated at 37°C for 2 hr, mixed with soft agar, and poured onto supplemented L-agar containing tetracycline (12.5 μg/ml), diaminopimelic acid (100 μg/ml), and thymidine (40 μg/ml). Subsequent incubation was at 37°C for 2 days.

Assays. Radioactive assays for fibrinolysis were carried out according to a published method (20). Radioimmunoassays in plastic-well microtiter plates and with CNBr-activated paper were done as described by Clarke *et al.* (21).

Physical and Biological Containment. The National Institutes of Health guidelines for research involving recombinant DNA molecules were followed; manipulations with recombinant DNA organisms were done under P2-EK2 containment.

RESULTS

The experimental strategy for the cloning of the human urokinase gene and its expression in *E. coli* consisted of the following steps. Initially, we isolated total RNA from human fetal kidney cells that produce urokinase and isolated the urokinase mRNA from the poly(A)-containing mRNA by sucrose density gradient centrifugation. In tissue culture studies we observed that a large species (150,000 daltons) of urokinase was produced early in the synthesis. Thus, the urokinase mRNA would be so large that we should be able to separate it from the bulk of the other cellular mRNAs. The urokinase-specific mRNA was detected by translation in cell-free protein synthesis systems as described above and by immunoprecipitation of newly synthesized urokinase protein with specific urokinase antibody. Indeed, urokinase mRNA was found to be larger than 28 S. To produce hybrid plasmids with pBR322, double-stranded cDNA was synthesized from the purified urokinase mRNA and inserted at the *Pst* I site with poly(dC)·poly(dG) connectors. This cloning protocol allows one to regenerate the *Pst* I restriction site, to insert the cDNA into an actively transcribed gene, and to eliminate the *bla* gene so that one can select for clones containing hybrid plasmids. The transformants of strain χ1776 that contain the hybrid plasmids are tetracycline resistant and ampicillin sensitive.

We then detected the expressed products of the urokinase gene in the clones containing hybrid plasmids by using a solid-support radioimmunoassay for urokinase antigens. Finally, the properties of the protein product of the recombinant DNA were compared with those of human urokinase.

Initial Detection of Urokinase-like Protein in Transformants. A total of 32 tetracycline-resistant transformants was obtained from 0.15 nmol of the hybridized DNA mixture. Of these, four were ampicillin sensitive and contained inserts in their plasmids. Analyses of *Pst* I digests of the plasmids by gel electrophoresis revealed that the plasmids from three transformants had an insert of approximately 4.2 kilobase pairs whereas the plasmid from the other transformant contained a much smaller insert. Transformants χ1776–pABB19 and χ1776–pABB26 were grown in liquid medium and cell lysates were prepared (22). Antigens in the lysates were covalently bound to CNB-activated paper (21). Urokinase-like antigens were detected by their reaction with ^{125}I-labeled anti-urokinase antibody (21). The lysates from both transformants showed positive immunoreactivity compared to the control, strain χ1776–pBR322 (Fig. 1). The weak positive reaction of the transformants, compared with known amounts of type S_1 urokinase applied in the paper as a positive control, suggested that the transformants produced small amounts of urokinase.

Isolation and Purification of Urokinase-like Protein. Benzamidine is an inhibitor of urokinase and other serine proteases. When it is coupled to Sepharose, a protease-specific affinity column is formed (23). Cell lysates of χ1776–pBR322 or χ1776–pABB26 were applied to a benzamidine-Sepharose affinity column. Each eluted fraction was assayed for urokinase by radioimmunoassay in plastic microtiter plates (21). Fig. 2 shows the results of such an experiment. A single positive peak was observed for strain χ1776–pABB26, which clearly indicates the presence of urokinase-like materials. Strain χ1776–pABB19 showed a similar result (data not shown). Strain χ1776–pBR322 in a parallel experiment did not show any protein product that reacted with antibody against urokinase.

Plasminogen Activator Activity of Proteins Encoded by Plasmids pABB26 and pABB19. To test if the protein products made by strains χ1776–pABB19 and χ1776–pABB26 could activate human plasminogen, the lysates were also analyzed in a plas-

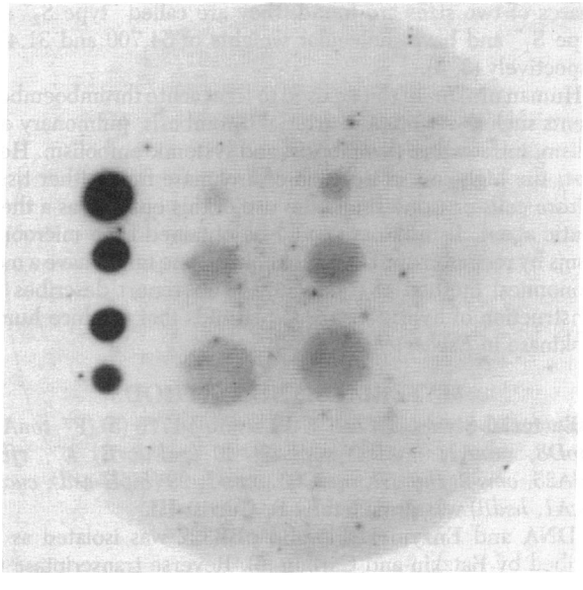

FIG. 1. Detection of urokinase-like material in lysates of transformants. Transformants χ1776–pABB19 and χ1776–pABB26 and the negative control χ1776–pBR322 were grown overnight and cells were centrifuged and lysed according to a published procedure (22). Aliquots (1, 5, or 10 μl) of the lysates were spotted directly on a CNBr-activated paper disc. Urokinase antigens were detected as described by Clarke *et al.* (21). From left to right, column 1 from top, 5 ng, 1.25 ng, 0.3 ng, and 0.08 ng of urokinase standard; column 2 from top, 1, 5, and 10 μl of χ1776–pABB19; column 3 from top, 1, 5, and 10 μl of χ1776–pABB26; column 4 from top, 1, 5, and 10 μl of the control transformant, χ1776–pBR322.

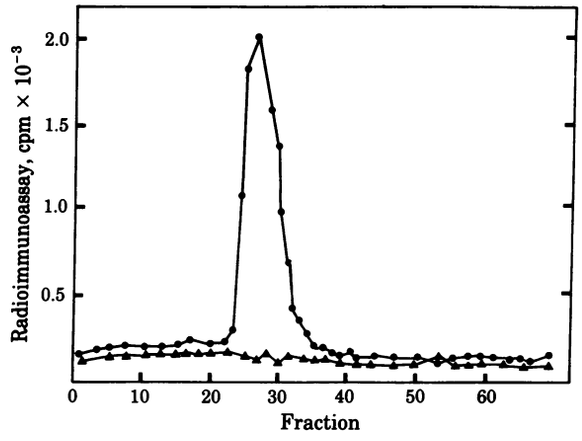

FIG. 2. Benzamidine affinity column chromatography of urokinase-like material. One-liter cultures of *E. coli* χ1776 containing either pABB26 or pBR322 were grown overnight, and the cells were collected by centrifugation and lysed as described in Fig. 1. The lysate was dialyzed against starting buffer (0.1 M sodium phosphate, pH 7.0/0.4 M NaCl). After dialysis, the lysate was loaded on a benzamidine-Sepharose column (23), washed thoroughly with the starting buffer, and then eluted with 0.1 M sodium acetate, pH 4.0/0.4 M NaCl. Fractions were collected and aliquots were taken for radioimmunoassay in plastic-well microtiter plates. ●, χ1776–pABB26; ▲, χ1776–pBR322.

minogen-dependent radioactive fibrinolysis assay (20). Because crude bacterial lysates interfered with the assay, only samples purified by affinity chromatography were used. The negative control, χ1776–pBR322, was devoid of any enzymatic activity that activated human plasminogen (Table 1). In contrast, both χ1776–pABB19 and χ1776–pABB26 clearly exhibited enzyme activities for human plasminogen activator.

Table 1. Plasminogen activator activity in bacterial transformants

Sample	Antisera	cpm	Milli-units	Activity remaining, %
Background	None	798	—	—
Urokinase standard	None	7,564	35.0	100
	Anti-urokinase	1,630	2.5	7
	Normal rabbit serum	3,458	12.0	35
χ1776(pBR322)	None	952	0	—
χ1776(pABB19)	None	18,560	175.0	100
	Anti-urokinase	1,886	6.0	3.4
	Normal rabbit serum	17,651	156.0	90
χ1776(pABB26)	None	10,887	50.0	100
	Anti-urokinase	1,479	2.3	4.6
	Normal rabbit serum	5,127	23.0	46

Plasminogen activator activity was measured by an ^{125}I fibrinolysis assay modified after a published method (20). Microtiter wells were coated with ^{125}I-labeled fibrinogen (2 μg/10^5 cpm per well) and clotted with plasmin-free thrombin prior to assay. Each assay well contained 0.1 M Tris·HCl (pH 8.1), 0.025% human serum albumin, and 2.5 μg of plasmin-free plasminogen per ml in a total volume of 70 μl. The range of the assay was from 0.05 Ploug unit/ml to 10 units/ml and the assay could detect as little as 0.002 unit. The relationship between radioactivity and biological activity was logarithmic. For antibody-mediated clearance of enzyme activity, 10 μl of a 1:10 dilution of the indicated antiserum was added to 25 μl of sample solution. After 60 min on ice, the immune complexes were cleared by adsorption to 25 μl of 10% (vol/vol) suspension of *Staphylococcus aureus* according to a published method (24).

The immunochemical relatedness of urokinase and the above enzyme activities was also studied by the loss of fibrinolytic activity after immunoprecipitation with antiserum to urokinase. Immunoprecipitation with anti-urokinase and *Staphylococcus aureus* removed 95% of the activity of urokinase or of the enzyme produced from the hybrid plasmids. Therefore, both molecules share the same antigenic determinants. Normal rabbit serum also was inhibitory, but the extent of inhibition was limited to 25 milliunits of activity regardless of how much enzyme was present in the assay. Plasminogen activator activity derived from urokinase or from a product expressed by the hybrid plasmids behaved identically in this respect. Normal serum is known to be inhibitory in this type of assay (20).

Molecular Species of Urokinase-like Material Expressed by χ1776–pABB26. Samples eluted from the affinity column were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and then transferred by blotting onto CNBr-activated paper. Lysates of χ1776–pABB26 exhibited five protein bands that reacted with urokinase antibody (Fig. 3). The positions

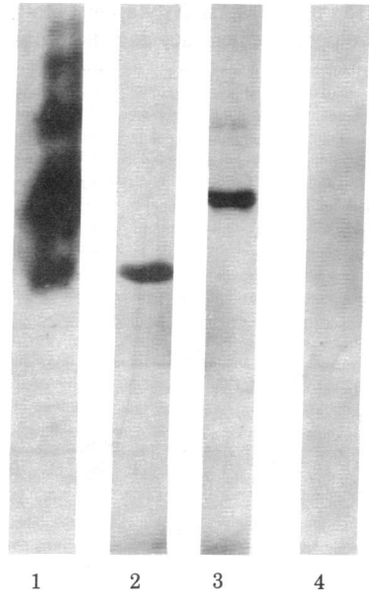

FIG. 3. Electrophoresis and filter-affinity-transfer analyses of urokinase-like material. Fractions eluted from the affinity column were dialyzed against water and lyophilized. Samples were prepared in 0.1% NaDodSO$_4$/60% (vol/vol) glycerol/10 mM Tris glycine, pH 8.6. Electrophoresis with a 6–30% polyacrylamide gradient gel (25) was run at 10 mA for 18 hr. Proteins of known molecular weights [type S_2 and type S_1 urokinase (4), phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin] were coelectrophoresed and stained with Coomassie blue for comparison. After electrophoresis, individual lanes were cut out and immersed in phosphate-buffered saline/0.5% Nonidet P-40 for 30 min. The gel was then transferred to a blotter (Whatman 3MM paper) soaked with the same buffer. Proteins were transferred from the gel to strips of CNBr-activated paper overnight. After the transfer, paper strips were incubated for 4 hr in 0.1 M glycine/0.1% bovine serum albumin and then washed thoroughly with 5% calf serum in phosphate-buffered saline. ^{125}I-Labeled urokinase antibody (1.5 × 10^6 cpm) in 3 ml of 25% calf serum/10 mM Tris, pH 7.2/0.15 M NaCl was added to each paper strip in individual sealed plastic bags. Overnight incubation was carried out at 37°C, and the strips were washed with the calf serum solution, blotted dry, and analyzed by autoradiography. Lanes: 1, χ1776–pABB26; 2, type S_1 urokinase; 3, type S_2 urokinase; 4, χ1776–pBR322. Lanes 2 and 3 were visualized with Coomassie blue.

of the bands corresponded to proteins of molecular weights of 150,000, 125,000, 87,000, 52,000, and 32,000. The predominant band was that of the 52,000 species. The smallest two of the products resemble types S_1 and S_2 urokinase in size. The negative control χ1776–pBR322 lysate did not show any band immunoreactive with urokinase antibody.

In addition to reacting with urokinase antibody, some of these proteins could be shown to manifest plasminogen activator activity. Extracts were made from χ1776 containing either pABB26 or pABB19. Proteins from the periplasmic space of these strains were electrophoresed on a $NaDodSO_4$ gel. The gel was sliced, and plasminogen activator activity was assayed in each fraction. Both strains exhibited a major urokinase activity peak at molecular weights of 52,000 and 125,000 (Fig. 4). There were also minor peaks between them. These enzymatically active proteins correspond to some of the bands of protein that reacted with the urokinase antibody (Fig. 3). The major antigenic component (darkest band in lane 1, Fig. 3) corresponds to the 52,000-dalton peak with major activity seen in Fig. 4. In Fig. 3, the urokinase found in the whole cell is displayed, not just that in the periplasmic space. Enzyme activity from the cytoplasm alone (data not shown here) was less than that found in the periplasmic space and included a major species of 48,000 daltons and very little activity in the 100,000-dalton region. Thus, most of the urokinase activity appears to be excreted by the cell into the periplasmic space and then into the medium.

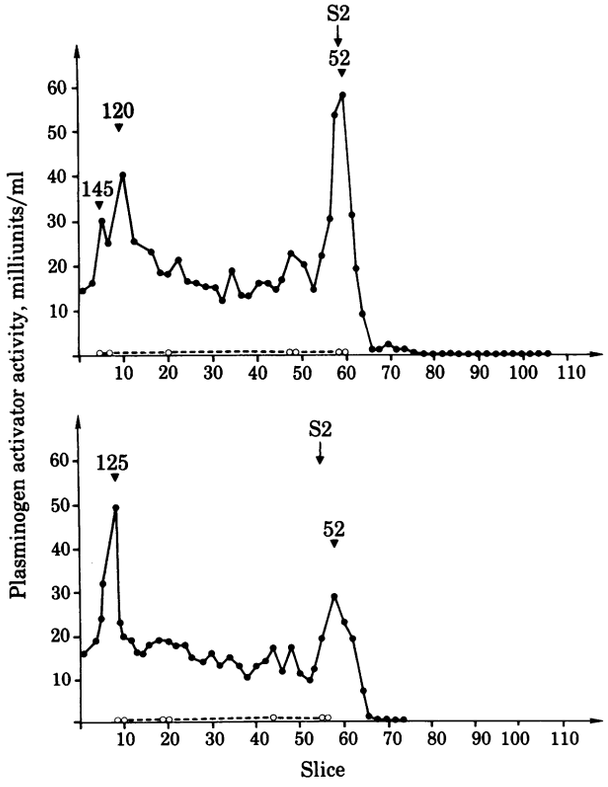

FIG. 4. Plasminogen-activator activity found in the periplasmic space of χ1776–pABB19 (*Upper*) and χ1776–pABB26 (*Lower*). Overnight cultures of these strains containing the hybrid plasmids were prepared and proteins in periplasmic space were isolated as described by Seeburg *et al.* (22). The proteins were run on a 0.1% $NaDodSO_4$/7% acrylamide gel as described by Laemmli (8). One-millimeter slices of gel were soaked in 100 vol of 0.1 M Tris, pH 8.1./0.025% human serum albumin. The eluates were then assayed for plasminogen-activator activity by the radioactive fibrinolysis assay (20). o----o, Without plasminogen. Numbers indicate molecular weight $\times 10^{-3}$; S2 is type S_2 urokinase marker.

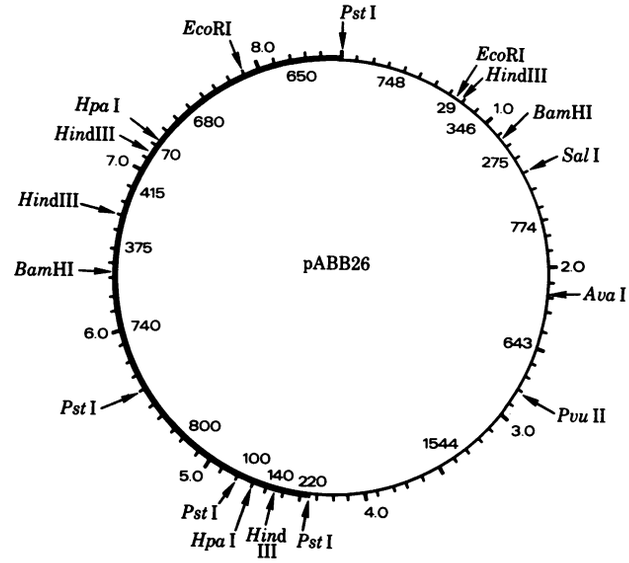

FIG. 5. Restriction map of hybrid plasmid pABB26. DNA fragments after digestion were separated on agarose gels in 40 mM Tris acetate, pH 7.8/1 mM EDTA. *Hin*dIII-digested λ DNA and known fragments of pBR322 DNA were used as molecular weight standards (26). The numbers outside of the circle are DNA lengths in kilobases; the numbers inside the circle are DNA lengths between two restriction sites (±10%). The thicker line of the circle represents the urokinase cDNA insert.

Restriction Map of pABB26. This recombinant plasmid, which carries the coding sequence for plasminogen activator at the *Pst* I site of pBR322, was characterized by restriction endonuclease digestion. The plasminogen-activator gene insert contains no site for *Sal* I, *Ava* I, or *Pvu* II, one site for *Eco*RI and *Bam*HI, two sites for *Hpa* I and *Pst* I, and three sites for *Hin*dIII (Fig. 5). The total length of pABB26 DNA is about 8550 base pairs, with an insertion (thick line in Fig. 5) of about 4200 base pairs.

DISCUSSION

The evidence presented here indicates that we have isolated hybrid plasmids containing a DNA sequence that specifies a plasminogen activator related to human urokinase and that *E. coli* transformed by these plasmids produces a biologically active enzyme. The protein product shares antigenic determinants with human urokinase, binds to a benzamidine-Sepharose column as does urokinase, has molecular sizes similar to those of urokinase, and has enzyme activity as a human plasminogen activator. Thus, these plasmids produce urokinase-like material. The products are among the largest human proteins expressed to date by hybrid plasmids in *E. coli*.

Preliminary results in which the proteins encoded by the hybrid plasmid were pulse-labeled in mini-cells (27) showed similar rates of expression for the plasminogen activator and for other gene products encoded in pABB26 DNA sequences. The amount of plasminogen activator produced in overnight cultures is low, but the low yield from the cell may be the consequence of protein degradation (28) or loss by excretion into the medium. The major portion of the urokinase produced by the hybrid plasmids seems to be located in the periplasmic space, and this distribution indicates that the bulk of the protein may be excreted into the medium. In that case, the bulk of the protein produced in an overnight culture would be in the medium. Such a result would be consistent with the facts that the urokinase cDNA is inserted into the *bla* gene whose product is also an

excreted protein and that human urokinase itself is an excreted protein. Consequently, hydrophobic leader sequences to a bacterially produced protein might be provided for by the attached promoter-proximal sequences of the *bla* gene. If leader sequences in eukaryotic proteins can be functional in a prokaryote, then perhaps the urokinase-specific leader sequences also play a role in excretion.

It is interesting that the molecular sizes of urokinase expressed by the hybrid plasmids are similar to those produced in tissue culture (unpublished data). The presence of a large polypeptide (molecular weight, 150,000) and of smaller active species can be interpreted as processing of the largest form by autocatalytic cleavage of urokinase or by peptide cleavage by a protease from *E. coli*. This interpretation assumes that the active enzyme is formed from precursors. On the other hand, perhaps these proteins result from different transcripts of the urokinase gene, all of which contain some of the same protein sequence, as is the case with the large and small tumor antigens of simian virus 40.

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