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Microinjected Xenopus oocytes synthesize active human plasminogen activator

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ABSTRACT

Induction of synthesis of the protease plasminogen activator (PA) by hormones, oncogenic viruses and tumor promoters occurs at the transcription level. A novel bioassay for PA messenger RNA was developed to study the regulation of PA synthesis and the genetic elements involved in it. Poly(A)-containing RNA from HEp-3, a PA-rich tumor of human origin, was found to direct the synthesis of a new proteolytic activity when microinjected into *Xenopus* oocytes. Newly synthesized protease can be detected within a few hours after microinjection of minute quantities of unfractionated mRNA. The new enzymatic activity is indistinguishable from human PA: it is absolutely dependent on human plasminogen; it is neutralized by serum raised against urokinase, the human urinary PA; and it comigrates with urokinase and HEp-3 PA in gel electrophoresis, exhibiting a molecular weight of 60,000.

INTRODUCTION

Plasminogen activator (PA) is a highly specific serine protease which converts the inactive zymogen plasminogen to the active trypsin-like protease plasmin (see ref. 1 for review). PA is closely correlated with inflammation, cell migration and tissue remodeling as well as with cellular transformation, tumorogenicity, metastasis and tumor promotion (see refs. 2,3 for review; 4,5). The enzyme can be modulated in a variety of vertebrate cell types by physiolocial inducers such as hormones and/or cyclic AMP (2-6), and also by non-physiological inducers such as oncogenic viruses (2,3), tumor promoters (7-10) and physical and chemical DNA damaging agents (11). Experiments with inhibitors of macromolecular synthesis indicate that regulation of PA synthesis occurs at the level of transcription (9-13), and that RNA synthesis is also required for the suppression and deinduction of PA synthesis (9,12-14). Thus, a study of PA at the level of messenger RNA (mRNA) is necessary for the understanding of the variety of mechanisms controlling its genetic expression. Such a study has not been carried out so far. A prerequisite for this is an assay to monitor PA mRNA.

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Assays of mRNAs, frequently based on the identification of specific translational products synthesized *in vitro*, permit only the detection of relatively abundant species of mRNA. Difficulties in detecting scarce mRNA species thus make difficult the study of genetic elements coding for rare cellular proteins of known functions, and also the investigation of mechanisms which regulate their transcription. Missing, and particularly desirable, are assays for rare mRNA sequences, which direct the synthesis of regulatory proteins such as receptors and enzymes. To this class belongs the mRNA for PA, since PA synthesis is estimated to comprise only a small fraction of the cellular protein synthesis, even upon maximum induction, when enzyme levels increase by two orders of magnitude (8,9).

We developed a method to identify and quantify PA mRNA, based on its ability to direct the synthesis of a biologically active polypeptide product. The detection of such a product is feasible due to the unique sensitivity of the assays for PA activity, which compensates for the low level of PA mRNA. This high sensitivity is gained by measuring the proteolytic activity of the reaction product, plasmin, rather than that of PA itself (15). The translation of PA mRNA has been carried out by *Xenopus* oocytes, which have been extensively used as a translation system for a variety of microinjected mRNAs since its first introduction by Gurdon and colleagues (16). *Xenopus* oocytes have been shown to efficiently perform translation, processing and various post-translational modifications; in the case of mRNAs for secretory proteins, oocytes carry out also the secretion of the correct translational products (see refs. 17,18 for review). Moreover, mRNAs which direct the synthesis of human (19) and mouse (20) interferon, of mouse β-glucuronidase (21) and of human oligo-isoadenylate synthetase (22) have been shown to be translated in oocytes into biologically active products. We now report that *Xenopus* oocytes synthesize and secrete proteolytically active human PA in response to the injection of mRNA from a human carcinoma.

MATERIALS AND METHODS

Growth of HEp-3 tumors

A HEp-3 tumor grown in a chick embryo (23) was further propagated in nude mice (ICR, the Weizmann Institute animal facilities) by subcutaneous injections of tumor pieces. Subcutaneous tumors were removed (0.2-0.8 gr/mouse) 2-3 weeks later and transferred immediately into liquid nitrogen.

Preparation and analysis of poly(A)-containing RNA

RNA was extracted from HEp-3 tumors as previously described (24), with minor modifications. DNA and tRNA were quantitatively removed from RNA preparations by sodium acetate wash of the ethanol precipitates, and poly(A)-containing RNA was prepared by oligo(dT)-cellulose chromatography at 4°C (25). The electrophoretic migration profiles of 1-3 μg RNA samples were determined on 1% agarose gels (80x70x0.5 mm) following staining with "Stainsall" (Sigma). Transparencies were scanned in a Gilford 2400S spectrophotometer.

Microinjection of RNA into *Xenopus* oocytes

Adult *Xenopus laevis* females were obtained from the South African Snake Farm (Fish Hoek, South Africa). The toads were anaesthesized by cooling in ice, ovarian lobes were removed and individual stage 6 oocytes were selected.

Single oocytes were microinjected with amounts of RNA specified in the legends and incubated in groups of 10 at 21°C in modified Barth medium (2) for the indicated time. Control oocytes were injected with the same volume of Barth medium (16) and incubated similarly.

Fibrin plate assay for plasminogen activator

Extracts of oocytes were prepared in 100 μl of 0.5% Triton X-100 in H_2O with a teflon-glass homogenizer. PA activity was assayed in the fibrin plate assay as described (26) in the presence of purified human plasminogen. Briefly, 3 and 6 μl samples of each extract were added to wells containing insoluble ^{125}I-labeled fibrin and incubated at room temperature. Proteolysis was monitored by measuring the production of soluble iodinated peptides after 7, 10 and 14 hours. Urokinase (UK, Leo Pharmaceutical Products, Denmark) was applied as a standard (0.25 to 2 Ploug milliunits per well). Control samples from which plasminogen was omitted did not show any fibrinolytic reaction, indicating an absolute dependence on plasminogen of the activity tested.

RESULTS AND DISCUSSION

RNA was isolated from the human established epidermoid carcinoma HEp-3 (27), which is rich in PA (5), and fractionated by oligo(dT)-cellulose chromatography into the polyadenylated and nonadenylated RNA (25). The poly(A)-containing RNA migrated on an agarose gel as a broad peak between 1000 and 5000 nucleotides in length. This fraction induced the synthesis of active PA when microinjected into *Xenopus* oocytes, while the nonadenylated RNA did not enhance PA activity above the small background level observed with control

oocytes (Fig. 1). The poly(A)-containing RNA was efficiently translated *in vitro* in the reticulocyte lysate cell-free system (29). About 1% of the TCA-insoluble labeled translation products could be precipitated with antibodies to human PA which were complexed with goat anti-rabbit serum. No product with enzymatic activity could, however, be obtained in the *in vitro* system, probably due to the lack of post-translational modification. Supplementation of the reticulocyte system with microsomal fraction from dog pancreas was not sufficient to allow the detection of active PA.

The intracellular appearance of the new proteolytic activity could clearly be detected in oocytes as early as 4 hours post-injection, similar to the early appearance of globin (25) and interferon (29). The level of PA in oocyte extracts tapers off around 10 hours post-injection (Fig. 2). The fibrinolytic activity of the newly synthesized enzyme was absolutely dependent on the addition of human plasminogen to the reaction mixture, indicating that it belongs to the class of plasminogen activators. The low level of fibrinolysis detected by control oocytes was also plasminogen dependent (Fig.2).

Increase in PA activity was observed with increasing amounts of injected mRNA (Fig. 3). Activity could be detected following injection of as low as

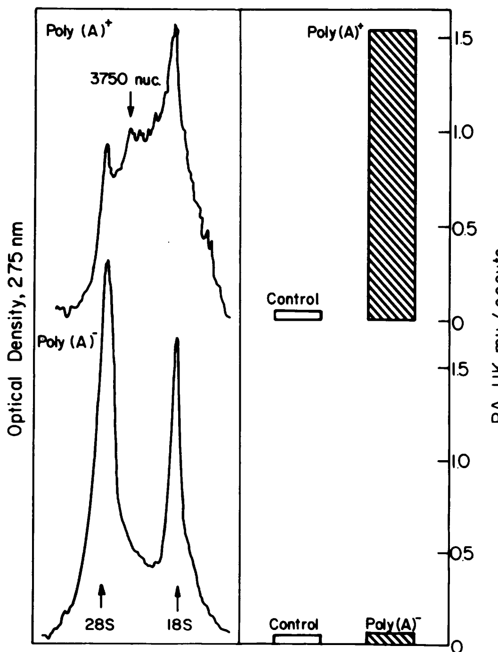

Figure 1: RNA fractions from HEp-3 tumors: Gel analysis and translation into active PA following microinjection into *Xenopus* oocytes. Poly(A)$^+$ and poly(A)$^-$ RNA were prepared and analyzed as described in Materials and Methods. Fifty nanograms of the two RNA fractions (1 mg/ml each) were microinjected into single oocytes, and 50 nanoliters of Barth medium were injected into control oocytes under standard conditions. Following 15 hrs of incubation, oocyte extracts were prepared and assayed for PA activity in the standard fibrin plate assay.

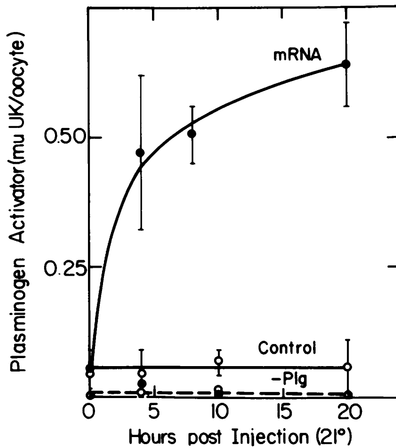

Figure 2: Time-course of PA synthesis in microinjected oocytes. Oocytes were injected with 50 ng of mRNA (•) or with Barth medium (o) as described in Materials and Methods, and incubated at 21°C for indicated times. Extracts of oocytes were prepared and assayed with (solid lines) or without (dashed lines) human plasminogen in the fibrin plate assay. No activity was obtained with any of the extracts when chicken plasminogen was employed. Circles represent mean values obtained from two separate experiments. Deviations from mean values are represented by the bars.

25 ng of total HEp-3 mRNA peroocyte, and 100 ng of mRNA appeared to saturate the translational capacity of the oocyte, as expected from previous reports(30). The two experiments shown in Fig. 3 differed in the background activity contributed by the oocytes, which were taken from two different frogs.

Newly synthesized PA was also detected at the level of single oocytes. PA activity appeared in a casein-containing overlay gel (31) as clear plaques on the opaque background of the casein. All oocytes microinjected with mRNA

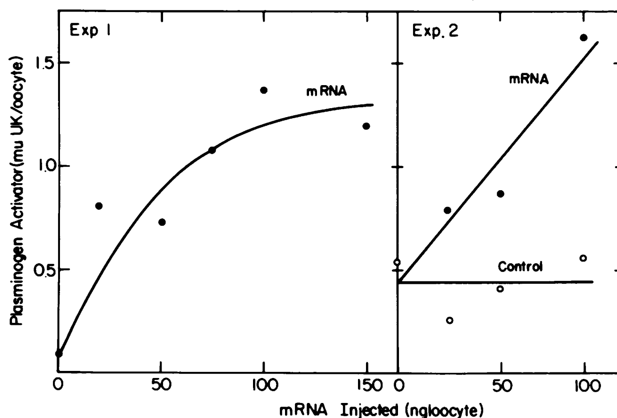

Fig. 3: Dose response to mRNA injection. Oocytes were injected with indicated quantities of poly(A)-containing RNA from HEp-3 tumors (•) or with Barth medium (o) and incubated for 15 hrs at 21°C. PA activity in oocyte extracts was assayed in the fibrin plate assay.

developed plaques of larger diameters than the plaques produced by control oocytes microinjected with Barth medium. Control plaques also exhibited a considerable variability in size (Fig. 4).

The human origin of PA synthesized by oocytes was verified by its immunoreaction with rabbit serum elicited against urokinase, the human urinary PA. Anti-urokinase serum which virtually neutralized completely urokinase as well as PA from an extract prepared from a HEp-3 tumor, also inhibited the activity of control oocytes (Fig. 5). In several experiments carried out with oocytes which displayed different levels of background activity, we have never observed a reduction of the endogenous proteolytic activity by anti-urokinase serum, whereas the newly synthesized activity has been neutralized in all cases. These results clearly show that the newly synthesized protease is immunologically similar to urokinase and HEp-3 PA, and is different from the proteases of the oocyte.

Measurement of the molecular weight of the newly synthesized PA further revealed similarity to urokinase and the HEp-3 enzymes. The electrophoretic analysis was carried out in polyacrylamide-SDS gels, which also contained plasminogen and a protein substrate (32). Following electrophoresis and removal of the SDS, the gel was first incubated and then stained, so that white bands representing proteolytic activity could be visualized on the dark protein background. The newly synthesized enzyme seen in oocyte lysate and also in the incubation medium comigrated with the 60,000 molecular weight band of both urokinase and HEp-3 PA (23), and the activity from all these sources was

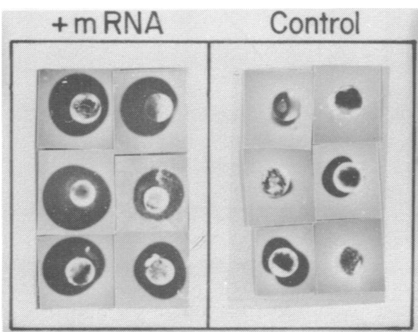

Figure 4: Synthesis of PA by single oocytes. Oocytes were injected with 100 ng of HEp-3 mRNA (left) or with Barth medium (right) as described for Fig. 1, and incubated in Barth medium for 2 hrs. They were then placed in a 30 mm dish and covered with 0.8 ml of agar-casein overlay, which contained 3 parts of Barth medium, 2 parts of 2.5% purified agar in Barth medium, 1 part of 8% solution of commercial nonfat milk powder (Carnation) and 20 μg/ml of human plasminogen. Following 15 hrs incubation at 21°C, oocytes were fixed with Dulbecco medium containing 10% pyridine and 2.5% glutaraldehyde. Photographs were taken on a dark field. No plaques could be observed in the absence of plasminogen.

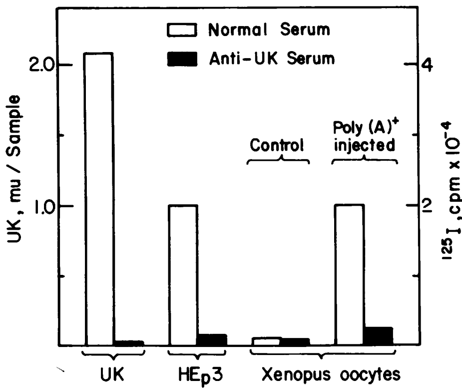

Figure 5: Neutralization of newly synthesized PA by rabbit anti-UK serum. Commercial urokinase (2 milliunits), extract prepared from a HEp-3 tumor or extract prepared from oocytes microinjected with HEp-3 mRNA (containing 1 milliunit UK) were reacted with rabbit antiserum (0.02 µl), raised against highly purified urokinase, or with rabbit control serum. The reaction mixtures of 50 µl contained phosphate buffered saline and 0.2% Triton X-100. Incubation was for 30 min at 37°C followed by 60 min at 4°C. 45 µl were then assayed for PA in the Linbro plate assay as described for Fig. 1. Control samples treated in the same way but in the absence of any serum gave results identical to those obtained with rabbit control serum.

absolutely plasminogen dependent. No activity of such migration properties could be seen in the samples derived from control oocytes, which secreted two plasminogen dependent bands of slower migration (Fig. 6).

The electrophoretic detection of PA, which is known to be a secretory

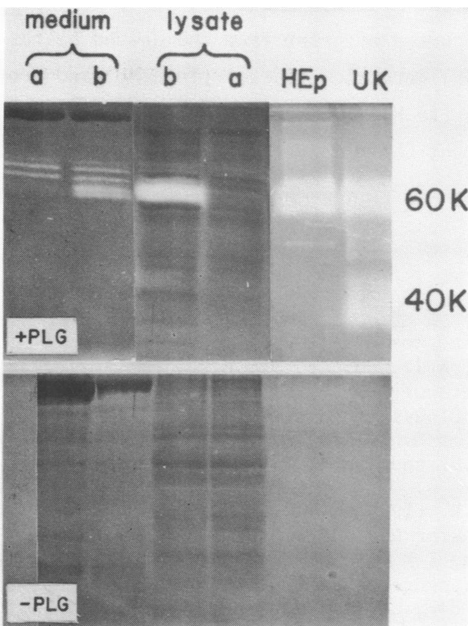

Figure 6: Electrophoretic analysis of newly synthesized PA in microinjected oocytes. Oocytes were microinjected with 100 ng of HEp-3 mRNA (b) or with Barth medium (a). Eight hours post-injection incubation media were collected and oocyte lysates were obtained from oocyte extracts (Materials and Methods) which were centrifuged to remove aggregates. Six µl of lysate and medium samples were zymographically analyzed for PA activity in SDS-polyacrylamide gel. Commercial urokinase and HEp-3 extract (about 0.5 Ploug milliunits each) were also analyzed. Analysis was as described by Huessen and Dowdle (29), except that gelatin was replaced by 0.1% commercial non-fat dried milk (Carnation) and the thickness of the gel was 0.5 mm. Upper gel -human plasminogen present (20 µg/ml). Lower gel-plasminogen absent.

enzyme (1), in oocyte medium, is in line with earlier findings showing that *Xenopus* oocytes secrete into the medium secretory products, like interferon (20,29) and milk proteins (33), but not non-secretory proteins such as histones or globin (33). Secretion of PA was also demonstrated in quantitative assays(26) In several experiments the secreted activities collected 20 hours post-injection were found to be 10 to 50% of the counterpart intracellular activities. Plasminogen independent proteases secreted by oocytes, which may degrade secreted PA, might account at least in part for this variability (Soreq and Miskin, submitted for publication).

The new bioassay for PA mRNA can now be utilized in studying PA regulation at the mRNA level following induction by hormones, oncogenic viruses and tumor promoters. Moreover, this bioassay can be used to monitor the enrichment of PA mRNA, which is a prerequisite for the construction of a cloned cDNA probe. Such a genetic probe can then be used to investigate the transcriptional and post-transcriptional regulation of PA synthesis and also to determine the number and structure of PA gene(s).

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REFERENCES

1. Christman, J.K., Silverstein, S.C. and Acs, G. (1977) in Proteinases in Mammalian Cells and Tissues, Barret, A.J., Ed., p. 91, Elsevier, Amsterdam.
2. Reich, E. (1978) in Biological Markers of Neoplasia: Basic and Applied Aspects, Ruddon, R.W., Ed., p. 491, Elsevier, Amsterdam.
3. Reich, E. (1978) in Molecular Basis of Biological Degradative Processes, Berlin, R.D., Hermann, M., Lepow, I.H. and Tanzer, J.M., Eds., p. 155, Acadmic Press, New York.
4. Ossowski, L., Biegel, D. and Reich, E. (1979) Cell 16, 929.
5. Ossowski, L. and Reich, E. (1980) Cancer Res. 40, 2310.
6. Wigler, M., Ford, J.P. and Weinstein, I.B. (1975) in Proteases and Biological Control, Cold Spring Harbor Conference on Cell Proliferation, Reich, E., Rifkin, D.B. and Shaw, E., Eds., p. 849, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

7. Wigler, M., DeFeo, D. and Weinstein, I.B. (1978) Cancer Res. 38, 1434.
8. Weinstein, I.B., Wigler, M. and Pietropaolo, C. (1977) in Origin of Human Cancer, Conferences on Cell Proliferation, Hiatt, H.H., Watson, J.D. and Winston, J.A., Eds., Vol. 4, p. 751, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
9. Wilson, E.L. and Reich, E. (1979) Cancer Res. 39, 1579.
10. Miskin, R., Easton, T.G. and Reich, E. (1978) Cell 15, 1301.
11. Miskin, R. and Reich, E. (1980) Cell 19, 217.
12. Wilson, E.L. and Reich, E. (1978) Cell 15, 385.
13. Rifkin, D.B., Beal, L. and Reich, E. (1975) in Proteases and Biological Control, Cold Spring Harbor Conferences on Cell Proliferation, Reich, E., Rifkin, D.B. and Shaw, E., Eds., p. 841, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
14. Vassalli, J-D., Hamilton, J. and Reich, E. (1976) Cell 8, 271.
15. Unkeless, J.C., Tobia, A., Ossowski, L., Quigley, J.P., Rifkin, D.B. and Reich, E. (1973) J.Exp.Med. 137, 85.
16. Lane, C.D., Marbaix, G. and Gurdon, J.B. (1971) J.Mol.Biol. 61, 73.
17. Lane, C.D. and Knowland, J. (1975) in The Biochemistry of Animal Development, Weber, R., Ed., Vol. II, p. 145, Academic Press, New York.
18. Marbaix, G. and Huez, G. (1980) in Transfer of Cell Constituents into Eukaryotic Cells, Celis, J.E., Graessmann, A. and Loyter, A., Eds., p.347, Plenum Press, New York and London.
19. Reynolds, F.H., Premkumar, E. and Pitha, P. (1975) Proc.Natl.Acad.Sci.USA 72, 4881.
20. Lebleu, B., Hubert, E., Content, J., De Wit, L., Brando, I.A. and De Clercq, E. (1978) Biochem.Biophys.Res.Commun. 82, 665.
21. Labarca, C. and Paigen, K. (1977) Proc.Natl.Acad.Sci.USA 74, 4462.
22. Shulman, L. and Revel, M. (1980) Nature 288, 98.
23. Ossowski, L. and Reich, E. (1980) Cancer Res. 40, 2300.
24. Soreq, H., Harpold, M., Wilson, M. and Darnell, J.E. (1980) Biochem. Biophys.Res.Commun. 92, 485.
25. Nudel, U., Soreq, H., Littauer, U.Z., Marbaix, G., Huez G., Leclercq, M., Hubert, E. and Chantrenne, H. (1976) Eur.J.Biochem. 64, 115.
26. Soreq, H. and Miskin, R., Brain Res., in press.
27. Toolan, H.W. (1954) Cancer Res. 14, 660.
28. Pelham, H.R.B. and Jackson, R.J. (1976) Eur.J.Biochem. 67, 247.
29. Soreq, H., Sagar, A.D. and Sehgal, P.B. (1981) Proc.Natl.Acad.Sci.USA 78, 1741.
30. Laskey, R.A., Mills, A.D., Gurdon, J.B. and Partington, G.A. (1977) Cell 11, 345.
31. Goldberg, A. (1974) Cell 2, 95.
32. Huessen, C. and Dowdle, E.B. (1980) Analytical Biochem. 102, 196.
33. Colman, A. and Morser, A. (1979) Cell 7, 517.