Determination of tissue-type plasminogen-activator mRNA in human and non-human cell lines by dot-blot hybridization

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A labelled cDNA clone was used in DNA–RNA hybridization on nitrocellulose filter paper (dot-blot technique) to detect and quantify mRNA for endogenous tissue plasminogen activator (PA) in cell extracts and samples of RNA purification runs. Although, for detection purposes, the assay was less sensitive than translation in *Xenopus* oocytes, it was at least as reliable and much more convenient for the purpose of quantitative determination. In particular, the technique was used to study the kinetics of PA mRNA formation in a human melanoma cell line (Bowes) after exposure to the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Incubation of the cells with TPA resulted in a 15–20-fold increase in cellular PA mRNA content. The effect was time- and dose-dependent: the increase in PA-specific mRNA was clearly visible as early as 4 h after initiation of TPA treatment in Bowes cells. It was blocked completely by pretreatment of the cells with actinomycin D, indicating that TPA caused enhancement of synthesis of PA mRNA rather than inhibition of PA mRNA degradation. The use of the nitrocellulose dot-blot technique also revealed that two non-human cell lines produce mRNAs which cross-react with the human PA mRNA, namely the mouse melanoma cell line B16 and the rat brain-tumour cell line, RT4-71-1. TPA was found to exert similar stimulatory effects on the synthesis of mRNAs in these cell lines as in Bowes cells.

INTRODUCTION

The human melanoma cell line, Bowes, secretes extrinsic tissue-type PA (Rijken & Collen, 1981). This PA is of particular interest because of its advantages in thrombosis treatment compared with urokinase and streptokinase. Previous studies from our laboratory have dealt with the isolation and the purification of centigram quantities of PA from Bowes cells (Collen *et al.*, 1982), its clinical value (Weimar *et al.*, 1981), and the isolation and characterization of the mRNA (Opdenakker *et al.*, 1982). Partial (Edlund *et al.*, 1983) and complete (Pennica *et al.*, 1983) cDNA sequences of tissue PA have been published. Also a single-step purification method by means of monoclonal antibodies directed against PA has been described (Nielsen *et al.*, 1983).

Few studies have been done on regulation of PA production. One of the multiple biological and biochemical activities of TPA, a potent tumour promoter (Mastro, 1982), is enhancement of production of urokinase-like PA (Wigler & Weinstein, 1976; Wilson & Reich, 1979; Crutchley & Maynard, 1983). This form of PA has been shown to be involved in metastasis (Ossowski & Reich, 1983). In Bowes cells, the regulatory effects of TPA on production of the different extrinsic tissue-type PA were putatively localized at the transcription level, as measured by mRNA-translation experiments (Opdenakker *et al.*, 1983) and PA protein determinations (Ashino-Fuse *et al.*, 1984). Cellular mRNA contents could only indirectly be determined by measurement of translation and secretion products. Furthermore, this test system relied on complex biochemical reactions, likely to be disturbed by impurities in the samples or by

experimental circumstances. In the present study we have used hybridization with a PA-specific DNA probe to determine directly amounts of PA mRNA. The technique was applied to study the regulation of PA mRNA by TPA, and to examine non-human cell lines for presence of mRNAs that cross-reacted with the human PA mRNA.

MATERIALS AND METHODS

Cells, culture media and materials

The human melanoma cell line (Bowes) and the rat brain-tumour cell line (RT 4-71-1) were obtained from Dr. D. B. Rifkin (Rockefeller University, New York, U.S.A.). They were cultured as described by Imada *et al.* (1978). The human osteosarcoma cell line MG 63 (Billiau *et al.*, 1977), the human larynx carcinoma cell line Hep-2 (American Type Culture Collection CCL23) and the rat melanoma cell line B16 (Wang *et al.*, 1980) were cultured in modified Eagle's minimum essential medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, non-essential amino acids and 1.35 mg of NaHCO₃/ml. All cell cultures were grown in 175 cm² stoppered plastic flasks (no. 3045; Falcon, Oxnard, CA, U.S.A.) at 37 °C to confluency in 100 ml of medium per flask. Before and between treatments of the cell cultures, they were washed with phosphate-buffered saline, supplemented with CaCl₂ and MgCl₂ (Opdenakker *et al.*, 1982). Treatment with actinomycin D (Serva, Heidelberg, W. Germany) and/or TPA (Sigma, St. Louis, MO, U.S.A.) was done in 20 ml of medium per flask. Actinomycin D was given in a dose of 1 µg/ml during

Abbreviations used: PA, plasminogen activator; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; poly(A)⁺ RNA, polyadenylated RNA; SSC, 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.4.

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30 min before other treatments. This dose inhibited the incorporation of radiolabelled uridine into RNA. Application of TPA was at different doses and time intervals as indicated. For optimal induction of plasminogen activator, we used a dose of 100 ng of TPA/ml for 4–6 h.

The radioactive isotopes were from Amersham International.

Extraction and measurement of RNA

The extraction procedure is a modification of a method previously described (Opdenakker *et al.*, 1982). It was adapted for larger numbers of samples, e.g. by decreasing the amount of cellular material. Confluent cultures of cells, growing in monolayers, were induced as described and suspended with phosphate-buffered saline without CaCl_2 and MgCl_2 but supplemented with 0.04% EDTA. Groups of treated and untreated cells were pelleted and lysed in a hypo-osmotic buffer, containing ribonucleosyl-vanadyl complexes to inhibit ribonuclease (Berger & Birkenmeier, 1979). Usually 10 ml of buffer was used per 10^8 cells. Total RNA was precipitated by addition of NaCl to 0.2 M and 2 vol. of ethanol overnight at -20°C . In some of the experiments it was subsequently fractionated by affinity chromatography on oligo(dT)-cellulose (Aviv & Leder, 1972), before the dot-blot assay and translation experiments. RNA was measured spectrophotometrically (A_{260}/A_{280}). Interference by detergents was excluded (Osterburg *et al.*, 1975). Since the vanadyl complexes, present in the total RNA preparations but not in purified RNA, could quench the A_{260} by up to 1%, only relative comparisons between different groups of total RNA preparations from extraction experiments with one vanadyl-complex stock solution were made.

Translation in oocytes and assay of extrinsic tissue-type plasminogen activator

RNA from different cell cultures was extracted and purified, and poly(A)⁺ RNA was injected and translated in *Xenopus laevis* oocytes, as described by Opdenakker *et al.* (1982). After 48 h incubation the supernatant fluids were tested for PA activity with the fibrin-plate method (Hoylaerts *et al.*, 1981). All measured activities were seroneutralizable with an affinity-purified antibody (given by Professor D. Collen, Center for Thrombosis and Vascular Research, Leuven, Belgium), directed against tissue PA. Culture media from the different cell lines were also screened for PA activity before and after TPA induction.

Measurement of PA mRNA with the nitrocellulose dot-blot assay

Plasminogen activator mRNA has been cloned in plasmid pAT 153 (T. J. R. Harris, T. Patel, P. Stephens, J. Kenten, F. A. O. Marston, S. Little, J. S. Emstage, G. Opdenakker, G. Volckaert, W. Rombauts, A. Billiau & P. De Somer, unpublished work). One of the clones contains an insert of approx. 1260 base-pairs, corresponding to the 5'-half of the mRNA. CsCl-gradient-purified plasmid DNA from this clone was used as a probe for PA mRNA in the dot-blot assay. In this test equal amounts of the RNA preparations were denatured by heating for 5 min at 70°C and quickly cooling at 0°C and suspended in $15\times\text{SSC}$. A logarithmic predilution series was made in $15\times\text{SSC}$ in microtitre plates, obtaining 100 μl per dilution. After wetting the nitrocellulose membrane in

double-distilled water and presoaking in $20\times\text{SSC}$, the RNA dilution series were applied to the filter. To standardize our method the same TPA-induced and uninduced RNA preparations were included on each filter. After loading the RNA on to the filters, they were baked for 3 h at 85°C . Prehybridization and hybridization conditions were as described by Dobner *et al.* (1981), but glycine was omitted and dextran sulphate decreased to 5%. The hybridization was done with a nick-translated cDNA probe (10^5 c.p.m./filter) overnight at 40°C (Thomas, 1980). After washing and drying, the filters were exposed to X-ray films (Curix RPII; Agfa Gevaert, Mortsel, Belgium) with an intensifying screen at -70°C for 1–4 days (Laskey & Mills, 1977).

After exposure, the autoradiograms were scanned in a Titertek apparatus (Flow Laboratories, Irvine, Scotland, U.K.) with a 450 nm filter. Densitometries were expressed in linear laboratory-scanner units. The nitrocellulose membrane was subsequently cut in 96 parts, dissolved in Soluene-100 (Packard, Downers Grove, IL, U.S.A.) and counted for radioactivity. Radioactivity (Čerenkov) was expressed as c.p.m. Every result was expressed as the mean value of two totally independent determinations.

RESULTS AND DISCUSSION

Determination of PA mRNA content with nitrocellulose dot-blots

Bowes melanoma cells were treated for 4 h with 100 ng of TPA/ml. The cells were suspended and total RNA was extracted. After precipitation and washing, the RNA was diluted in $15\times\text{SSC}$ and bound to nitrocellulose filters. The filters were then hybridized for 20 h with a nick-translated probe of a PA-specific gene fragment. Since mRNA for PA is not abundant, two methods were compared for the calibration of relative amounts of specific mRNA. In the first approach (Fig. 1a) the radioactivity of each spot of the nitrocellulose filter was determined and plotted against mRNA quantities. Parallel semi-logarithmic sigmoidal plots were obtained. The dose-response curves for mRNA preparations from TPA-induced cells were displaced relative to those for mRNA from non-induced cells, allowing one to calculate that their content of PA-specific mRNA was about 10-fold higher. Alternatively, the results were analysed by densitometry of autoradiograms of the filters. Figs. 1(b) and 1(c) respectively show the autoradiogram and the corresponding densitometric results obtained from the same nitrocellulose membrane as that used for generation of the data plotted in Fig. 1(a). Again, dose-response curves of TPA-induced and non-induced mRNA preparations were parallel, indicating that the same mRNA species was being measured. Displacement of the curves again allowed one to calculate that the content of PA-specific mRNA was about 12-fold higher in TPA-induced preparations. The sensitivity of the densitometric method of analysis was approx. 10 times higher than that of the radiometric assay. Therefore this method was used in subsequent dot-blot experiments.

To verify the accuracy of the densitometric test, ten fractions after linear sucrose-gradient centrifugation of poly(A)⁺ RNA from uninduced Bowes cells were tested with the dot-blot method and by translation *in vitro* in oocytes. Only one fraction of RNA had sequences

Fig. 1. Determination of PA-mRNA content with nitrocellulose dot-blots

Bowes melanoma cells were treated for 6 h with 100 ng of TPA/ml (+TPA, ●) or left untreated (-TPA, △). Total RNA was extracted and a dilution series was spotted on to a nitrocellulose filter. The filter was hybridized with a radioactive cDNA probe of the coding region of PA. Specific mRNA was assayed by direct counting (a) or by densitometric scanning (c) of the autoradiogram (b), in which two rows are shown for each treatment to indicate reproducibility.

Fig. 2. Purification of PA mRNA on oligo(dT)-cellulose

Melanoma cells were treated for 6 h with 100 ng of TPA/ml (+: □, ■) or left untreated (-: △, ▲). PA mRNA in total RNA preparations (▲, ■) or after purification on oligo(dT)-cellulose [i.e. in poly(A)⁺ RNA; △, □] was determined with the dot-blot assay and expressed as arbitrary comparative laboratory-scanner units.

complementary to the PA probe. Only that same fraction could be translated in *Xenopus* oocytes into biologically active PA. The identity of this PA to tissue PA was confirmed with zymography, seroneutralization of biological activity and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (results not shown). Also the sedimentation coefficient of PA mRNA in the linear sucrose gradient was confirmed to be approx. 20 S (Opdenakker *et al.*, 1982; Edlund *et al.*, 1983). In a similar experiment TPA-induced and non-induced RNA were purified by affinity chromatography on oligo(dT)-cellulose. The eluted poly(A)⁺ RNA was precipitated and washed. Serial dilutions of total and poly(A)⁺ RNA were applied in comparable amounts on to nitrocellulose filters. The densitometries of TPA-induced and non-induced RNA and of total RNA versus poly(A)⁺ RNA are compared in Fig. 2. The affinity chromatography retained less than 5% of the total RNA, for both TPA-induced and non-induced RNA. The TPA-induced increase in content of PA-specific mRNA was also evident in the poly(A)⁺ RNA preparations. Fig. 2 also shows that the technique can be applied for reliable measurements of PA-specific mRNA contents in rather impure RNA preparations. Indeed, with total RNA preparations (which consisted of more than 95% of RNA molecules not coding for PA) no detectable quenching of PA mRNA sequences was observed.

Effect of TPA on mRNA amounts as studied with the dot-blot technique

The dot-blot technique was used for direct measurement of PA-specific mRNA in Bowes cells treated with different doses of TPA for different time intervals. The autoradiogram obtained in this experiment is presented in Fig. 3, showing that PA-specific mRNA content increased with TPA dose. The enhancing of TPA on PA-mRNA content was also time-dependent: it was already obvious after 4 h, still increased at 6 h and then decreased. This time-dependence was most pronounced

Fig. 3. Dose- and time-dependency of the TPA-effect as assayed with the dot-blot technique

Bowes cells were treated with 0, 1, 10 or 100 ng of TPA/ml for the indicated time intervals. Total RNA was extracted, spotted on nitrocellulose filters and hybridized with a PA-specific cDNA probe. After washing, the filter was exposed for 24 h.

Fig. 4. Autoradiogram showing the inhibitory effect of actinomycin D on TPA-induced increase in PA mRNA

Bowes melanoma cells were pretreated for 30 min with 1 µg of actinomycin D/ml (ACT D). The cells were then treated for 4 h with 100 ng of TPA/ml. Controls were not treated. Total RNA was extracted, and equal amounts (A_{260} units) were assayed for the presence of PA mRNA with the nitrocellulose dot-blot technique.

with the highest TPA doses. Although it was known from previous studies that TPA increases 2-fold the poly(A)⁺ RNA content in Bowes cells (Opdenakker *et al.*, 1983), total RNA content of TPA-treated cells equalled those of untreated cells (7 µg/10⁶ cells).

The TPA-induced increase in PA-specific mRNA contents could be due to increase in mRNA generation (transcriptional effect) or to post-transcriptional stabilization. Therefore groups of confluent monolayers of Bowes cells were treated with actinomycin D before TPA-dependent PA mRNA induction. Actinomycin D was applied at 1 µg/ml for 30 min. This dose was sufficient to inhibit rapid [³H]uridine incorporation into RNA by more than 97% (results not shown). Then the cells were washed with phosphate-buffered saline and induced with 100 ng of TPA/ml for 4 h. Control groups consisted of untreated cells. Fig. 4 shows that TPA treatment resulted in a more than 10-fold increase in

Table 1. Relative mRNA content of different cell lines

PA-mRNA content was measured by densitometric analysis of nitrocellulose dot-blots. On each nitrocellulose filter a standard RNA preparation from Bowes cells was included. Displacement of the linear part of the densitometric scans allowed expression of relative PA mRNA content as % of that in untreated Bowes cells. Abbreviation: N.D., not detectable.

Cell line	Relative PA-mRNA content (%)	
	Untreated	TPA-treated*
Bowes	100	1200
Hep-2	N.D.	N.D.
MG-63	N.D.	N.D.
B-16	18	30
RT 4-71-1	16	30

* Cells were treated for 4 h with 100 ng of TPA/ml.

PA-specific mRNA. The PA mRNA content of Bowes cells treated with actinomycin D alone equalled those of untreated cells. Moreover, the TPA-induced increase in specific RNA could be completely blocked by pretreatment with actinomycin D before TPA treatment. It was thus concluded that TPA exerts its PA-inducing effect at the level of mRNA generation, i.e. transcription.

Identification in non-human cell lines of mRNAs cross-reacting with the Bowes PA mRNA

Human PA activates dog (Bergmann *et al.*, 1983), rabbit (Matsuo *et al.*, 1981) and human plasminogen. Furthermore, since the PA isolated from a rat brain-tumour cell line could be neutralized by antibody prepared against the human extrinsic tissue-type PA (Bykowska *et al.*, 1981), the possibility existed that the human PA probe could be used for detecting PA-mRNA production in non-human cells. Confluent cultures of human osteosarcoma cells (MG-63) and laryngeal carcinoma cells (Hep-2), mouse melanoma cells (B16) and rat brain-tumour cells (RT-4-71-1) were treated for 4 h with 100 ng of TPA/ml or left untreated. RNA was extracted and equal amounts of total RNA were compared in the dot-blot assay. Table 1 shows the densitometric results of these autoradiograms; not all the cell lines tested produced detectable mRNA for PA. Thus MG-63 cells and Hep-2 cells showed no detectable PA mRNA with or without TPA treatment. The two rodent cell lines, however, expressed mRNAs that hybridized with the PA-specific DNA probe. In both cell lines TPA treatment resulted in an up to 2-fold increase in PA mRNA, and this increase could be completely blocked with actinomycin D pretreatment. The enhancing effect of TPA on PA-mRNA content in these cells was less than in the melanoma cell line. Thus the total RNA content was unaffected, but the poly(A)⁺ content increased up to 1.5-fold. The increase in specific PA mRNA in these rodent cell lines seems to be a general transcriptional effect of TPA, rather than a specific effect on the PA gene.

Translation techniques *in vitro*, such as translation in *Xenopus laevis* (Opdenakker *et al.*, 1983), rely on complex biochemical or biological systems which are likely to be

influenced by impurities in the assay samples as well as by experimental circumstances, i.e. conversion of pro-PA into PA (Andreasen *et al.*, 1984). The main advantage of the dot-blot technique over translation methods *in vitro* is that it measures specific mRNA directly without quenching of activity in rather impure preparations. Also the dot-blot technique is more rapid and convenient. Previous studies (Van Damme *et al.*, 1985; Content *et al.*, 1985) have shown that the RNA-extraction procedures described are well adapted for blotting studies and that the dot-blot technique is better than Northern-blot analysis from a quantitative viewpoint. Furthermore, application of recently described methods might allow enhancement of the sensitivity up to 100-fold (Zinn *et al.*, 1983).

The technique was used to monitor the purification of PA mRNA from Bowes cells: poly(A)⁺ RNA represents less than 5% of the total RNA in Bowes cells, and ultracentrifugation in sucrose gradients can yield up to 40-fold enrichment in PA-specific mRNA. The technique allowed us to confirm that TPA treatment caused a rapid, up to 10-fold, increase in PA mRNA content per A_{260} unit of poly(A)⁺ RNA (Opdenakker *et al.*, 1983). Moreover, this effect results from increased mRNA synthesis rather than from inhibition of mRNA degradation. This increase was also found in total RNA preparations and could completely be blocked by pretreatment of the cells with actinomycin D. Therefore these experiments provide a definitive proof that TPA acts primarily on transcription. This implies that specific mRNA enrichment by this transcriptional effect can be exploited for the cDNA cloning and study of TPA-induced mRNAs.

The technique allowed us to detect PA-specific mRNA in other than human cells. It is known that tissue PA of the rat (Bykowska *et al.*, 1981) cross-reacts serologically with human tissue PA, indicating that there may be considerable homology at the level of DNA and mRNA. This expectation was borne out by our finding that a rat brain-tumour cell line known to produce tissue PA and a mouse cell line (B16) known to produce an unidentified PA (Wang *et al.*, 1980) both contained mRNA sequences that reacted positively in the dot-blot assay. Conversely, several other tumour cell lines, including two human ones, did not contain such mRNA. The fact that PA mRNA of various animal species hybridizes with the human cDNA probes should facilitate the isolation of these mRNAs, e.g. by hybrid selection (Mertens *et al.*, 1983), as well as the subsequent cloning and nucleic acid sequencing. Expression of recombinant mouse or rat tissue-type PA could provide an animal model for the study of the role of extrinsic tissue-type PA in metastasis of PA-producing tumour cells and of the thrombolytic properties *in vivo*. Until now only animal model studies with the different urokinase-like PA have been documented.

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