Plasminogen Activators and Their Inhibitors in a Human Mammary Cell Line (HBL-100)

MODULATION BY GLUCOCORTICOIDS*

Nathalie Busso†§, Dominique Belin†‡, Christiane Faillly-Crépin$, and Jean-Dominique Vassalli†

From the †Institut d'Histologie et d'Embryologie and ‡Département de Pathologie, Centre Médical Universitaire, Geneva, Switzerland, and the §Institut de Recherches Scientifiques sur le Cancer, Villejuif, France

Culture of human mammary HBL-100 cells in the presence of dexamethasone, a synthetic glucocorticoid, resulted in opposite effects on the production of the two plasminogen activators (PAs): a decrease in urokinase-type PA (u-PA) and a concomitant increase in tissue-type PA (t-PA). Two PA-specific inhibitors, one related to that isolated from human placenta, were also produced by these cells; dexamethasone did not affect the production of either of these inhibitors. The glucocorticoid effects observed on PA enzymatic activities were associated with changes in PA mRNA levels. Experiments using inhibitors of RNA and protein synthesis suggested that the glucocorticoid-induced decrease in u-PA mRNA was a secondary event, requiring synthesis of new regulatory proteins; in contrast, the increase in t-PA mRNA appeared to be a direct effect on t-PA gene expression.

Plasminogen activators (PAs) are arginine-specific serine proteases that convert the zymogen plasminogen into plasmin, a trypsin-like protease of broad substrate specificity (for a recent review, see Ref. 1). There are two types of mammalian PAs, urokinase-type PA (u-PA) and tissue-type PA (t-PA). A comparison of their amino acid sequences (2, 3) as well as of the nucleotide sequences of their cDNAs (4, 5) indicates that t-PA and u-PA are the products of different but related genes.

Cellular production of PAs is believed to play an important role in the control of extracellular proteolysis. PA activity, which is usually increased during neoplastic transformation, is regulated in both normal and neoplastic tissues by a variety of hormones and other effectors (6–11).

Glucocorticoids have been reported to suppress PA activity in different tissues and cultured cells (12–21). These include u-PA-producing human cells such as polymorphonuclear leukocytes (14, 15), embryonic lung cells (16, 17), and kidney cells (17). Rodent u-PA activity exhibited by pituitary cells (18), macrophages (19), and mammary glands (20, 21) is also inhibited by glucocorticoids. The modulation of t-PA activity by glucocorticoids appears to be more variable. Although glucocorticoids suppress t-PA activity in rat pituitary (18) and hepatoma cells (22), they do not inhibit t-PA activity of bovine aortic endothelial (23) and human melanoma cells (17); in some cases, human acute myeloid leukemia cells show a stimulation of t-PA secretion in response to glucocorticoids (15).

It has been previously suggested that glucocorticoid regulation of PA activity involves transcriptional event(s) which subsequently lead to changes in enzyme biosynthesis (13, 14, 15, 16, 19, 20). This suggestion, which is based on experiments with RNA synthesis inhibitors such as actinomycin D, has not yet been confirmed by a direct measure of PA mRNA levels. Furthermore, the recent description of PA inhibitors (15, 16) has led to the concept that the fibrinolytic activity of cells reflects the balance between the activities of PAs and those of their inhibitors: thus, agents which alter overall PA activity may do so by changing the levels of PAs, of inhibitors, or both. For instance, it has been reported that glucocorticoids decrease the PA activity of rat hepatoma cells, through the induction of a PA inhibitor (27). The aim of this work was to explore the molecular mechanisms underlying glucocorticoid regulation of PA activity in a human mammary cell line, HBL-100.

MATERIALS AND METHODS AND RESULTS³

Characterization and Glucocorticoid Regulation of PAs Produced by HBL-100 Cells—To investigate the production of PAs and its hormonal regulation in HBL-100 cells, we first used a quantitative caseinolytic assay. Cells cultured for 24 h in serum-free medium contained and secreted a plasminogen-dependent proteolytic activity. The level of cell-associated PA activity was close to 1 Plough unit/mg of cellular protein. PA activity was also recovered in the culture medium and the rate of PA accumulation was 0.5 unit/mg of cellular protein/24 h. In the presence of 10⁻⁷ M dexamethasone, the cell-associated PA activity was decreased 3–10-fold. The level of

³ Portions of this paper (including "Materials and Methods," part of "Results," and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9600 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-364, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
extracellular PA activity was more variable, ranging from 50 to 100% of control values (data not shown).

To determine the molecular species of PAs produced by HBL-100 cells, we have electrophoretically and immunologically characterized both cell-associated and extracellular PAs (Fig. 2). Briefly, the samples were mixed with anti-u-PA, anti-t-PA or irrelevant IgG, and the immune complexes precipitated by the addition of fixed Staphylococcus aureus; aliquots of the supernatants were subjected to SDS-PAGE, and the gels were analyzed by zymography. The control samples from untreated cultures contained one major form of PA (M, 55,000, tracks 3 and 9) which co-migrated with purified human u-PA (not shown). This activity was specifically precipitated in the presence of anti-u-PA IgG (tracks 1 and 7), indicating that both cell-associated and secreted enzymes were mainly of the u-PA type. Faint bands of t-PA-related forms (M, 72,000 and >100,000) were also present in the conditioned medium of control cells (track 9). As expected from the results of the caseinolytic assay, addition of dexamethasone to HBL-100 cells decreased the cell-associated PA activity (compare track 6 with track 3); the residual PA in hormone-treated cells was indistinguishable from u-PA. Zymographic analysis of the culture medium from dexamethasone-treated cells revealed a more complex pattern (track 12). By selective immunodepletion of t-PA, we could establish that dexamethasone treatment decreased the u-PA content of the culture medium also (compare tracks 8 and 11). Concomitantly, dexamethasone markedly increased t-PA-related activities in the culture medium (compare the u-PA-depleted samples, tracks 7 and 10). Thus, total PA activity in the medium of dexamethasone-treated cells was the sum of t-PA- and of u-PA-related activities (track 12); whereas exposure to the hormone decreased u-PA, it also markedly increased t-PA. Therefore, it is not surprising that the overall plasminogen-dependent caseinolytic activity in culture medium could have been unaffected by dexamethasone in some experiments. The distribution of t-PA and u-PA between cell extract and culture medium was markedly different; indeed, in agreement with previous observations, most of the t-PA was recovered in the culture medium (42) whereas a substantial fraction of u-PA remained cell-associated (43), presumably because of its interaction with u-PA-specific cellular receptors (44).

The t-PA-related enzyme was present in two distinct forms in the culture medium of dexamethasone-treated cells, with apparent M, values of 72,000 and >100,000, respectively. The M, 72,000 form co-migrated with purified free t-PA (data not shown), while the higher M, form consisted of a complex between t-PA and a specific PA inhibitor as discussed below. The relative amounts of free and complexed t-PA varied in different experiments; this could account for the variable PA activity in the culture medium of dexamethasone-treated cells.

To determine whether induction of t-PA-related activities detected by zymography could be accounted for by increased synthesis of t-PA protein, cells were incubated with [35S] cysteine, and the culture medium was analyzed by SDS-PAGE and autoradiography after immunoprecipitation with anti-t-PA (Fig. 3, tracks 1–4). Two-chain t-PA (M, 35,000–40,000), one-chain t-PA (M, 72,000), and inhibitor-complexed t-PA (M, >100,000) were found to be biosynthetically labeled, and the amount of label associated with all the species was higher in dexamethasone-treated cells. Densitometric analysis of the autoradiogram indicated that some 2-fold more radioactivity was associated with the M, >100,000 species than with the M, 72,000 free enzyme. Even if the cysteine content of the inhibitor was as high as that of t-PA (i.e. 35 cysteine residues/molecule (3, 5)), the amount of t-PA in the complex would still be comparable to that in the free, M, 72,000, form. However, zymographic analysis (Fig. 3, tracks 5 and 6) of the same sample as that analyzed by immunoprecipitation in tracks 3 and 4 showed that relatively little catalytic activity was revealed at the position of the M, >100,000 complex. Thus, the specific enzymatic activity of the M, >100,000 form was low compared to that of the M, 72,000 form. This result is consistent with the conclusion that the high M, form is a relatively inactive enzyme-inhibitor complex.

**HBL-100 Cells Produce Two Different Inhibitors of PAs—**

The high M, , t-PA-related species revealed by zymography and by biosynthetic labeling consisted of a complex of the enzyme with the endothelial-type PA-specific inhibitor first described by Loskutoff et al. (25). Indeed, this complex was

---

**Fig. 2. Immunoprecipitation of PAs produced by HBL-100 cells in the presence and absence of dexamethasone (DEX).** Samples of cell extracts or culture media were mixed with IgG from rabbits immunized against human u-PA (tracks 1, 4, 7, and 10) or human t-PA (tracks 2, 5, 8, and 11) or from un.injected rabbits (tracks 3, 6, 9, and 12). After SDS-PAGE of the supernatants, the gel was analyzed by zymography. Zymograms of cell extracts and conditioned media were photographed after 4 and 8 h, respectively.

**Fig. 3. Tracks 1–4, biosynthetic labeling of secreted t-PA in presence and absence of dexamethasone (dex).** Cell cultures were preincubated for 8 h in DMEM containing 100 μg/ml of bovine serum albumin without (tracks 1 and 2) or with (tracks 3 and 4) 10−7 M dexamethasone, and labeled for 16 h in modified DMEM containing [35S]cysteine (50 μCi/ml) and 5% of the normal amount of nonradioactive cysteine, in the presence or absence of dexamethasone. Culture media were immunoprecipitated with anti-t-PA (tracks 1 and 3) and nonimmune IgG (tracks 2 and 4) by addition of fixed S. aureus. Immune complexes were eluted and electrophoresed under reducing conditions. The gel was treated with ENHANCE, dried, and fluorographed. Tracks 3 and 6, zymography of the dexamethasone-treated medium analyzed in Fig. 3, tracks 1–4. Culture medium was mixed with anti-u-PA IgG (track 5) to avoid the possible influence of u-PA present in the samples or with nonimmune IgG (track 6); after addition of fixed S. aureus and centrifugation, the supernatants were subjected to SDS-PAGE, and the gel was analyzed by zymography.
immuno-depleted by addition of anti-endothelial-type inhibitor antiserum to the culture medium (Fig. 4A, track 2); antibodies to a different PA-specific inhibitor, originally described in placental extracts (45), did not react significantly with the high M, enzyme form (track 1).

To investigate further the production of PA inhibitors by HBL-100 cells, we used two additional methods that allow the detection of free, noncomplexed, inhibitors. The same immuno-depleted samples as in Fig. 4A were subjected to SDS-PAGE; the gel was then analyzed by reverse autography on a casein substrate containing both u-PA and plasminogen. Inhibitory activity was revealed as a lysis-resistant band (Fig. 4B), at an apparent M, of approximately 50,000. This inhibitor was absent in culture medium after immuno-depletion with antiserum to the endothelial-type inhibitor (track 2), whereas antiserum to the placental-type inhibitor (track 1) did not remove the inhibitor detected in the assay. Thus, culture medium from dexamethasone-treated HBL-100 cells also contained unreacted endothelial-type PA inhibitor. A comparison with the culture medium of untreated control cultures indicated that the hormone did not alter the amount of this inhibitor as revealed by reverse autography (not shown). This assay did not provide evidence for the presence of placental-type inhibitor.

Another assay of protease inhibitors takes advantage of the formation of high M, covalent enzyme-inhibitor complexes upon addition of radiolabeled enzyme. M, 33,000 125I-u-PA was added to samples of cell extracts and culture media of HBL-100 cells. The samples were incubated with the two specific anti-inhibitor antisera previously used, and the immunosupernatants were subjected to SDS-PAGE. Autoradiography of the fixed gel (Fig. 5) revealed, in addition to the radiolabeled u-PA, a radioactive M, 73,000 band. Most of the u-PA-inhibitor complex was absent after immunoprecipitation by antiserum to the placental-type inhibitor (tracks 1, 4, 7, and 10). By quantitating the amount of 125I-u-PA recovered as a complex, we could estimate the concentration of the placental-type inhibitor to be approximately 300 ng/mg of cellular protein in cell extract and 50 ng/mg of cellular protein in culture medium; these values were not significantly different in control or dexamethasone-treated cells.

In conclusion, HBL-100 cultures contained at least two different PA-specific inhibitors. The endothelial-type inhibitor present associated with t-PA in the form of a high M, complex, and in the M, 50,000 form reported for the free molecule after SDS-PAGE (25); however, little if any functional endothelial-type inhibitor could be revealed by addition of exogenous labeled u-PA, suggesting that the culture medium contained mostly the latent, SDS-activatable inhibitor (47). Placental-type inhibitor was also demonstrated in these cultures. Finally, dexamethasone did not alter the levels of either inhibitor.

**Analysis of u-PA and t-PA mRNA Content**—In view of the complex pattern of enzymes and inhibitors in cultures of HBL-100 cells, determination of PA mRNA levels appeared as the only way to provide a quantitative analysis of the effect of dexamethasone on the expression of the two PA genes. To measure mRNA content, we used total cellular RNA to rule out possible changes in polyadenylation levels; the RNAs were hybridized to single-stranded cRNA probes. Taking into account the length and the specific activity of the probes we estimated that both u-PA and t-PA mRNAs in control cultures were present at concentrations of 0.1–0.3 pg/μg of total cellular RNA. The effect of dexamethasone on u-PA mRNA level was measured at various times after the addition of the drug (Fig. 6A). A 2-fold decrease in u-PA mRNA content was detected after 3 h of dexamethasone treatment and was maintained for at least 17 h in the presence of the glucocorticoid. We also observed a time-dependent accumulation of t-PA mRNA after addition of dexamethasone (Fig. 6B). The amount of t-PA mRNA was 2-fold higher after 4 h and then remained constant for at least 17 h.

To determine whether these changes in PA mRNA levels resulted from a direct effect of the glucocorticoid on expression of the two PA genes, we pretreated cells for 30 min with cycloheximide, a protein synthesis inhibitor, before addition of dexamethasone. Cycloheximide alone had a marked effect on u-PA mRNA content, increasing it up to 7-fold (Fig. 7, track 3); this suggested that inhibition of protein synthesis leads to a stabilization of u-PA mRNA. Addition of dexamethasone to cycloheximide-treated cultures resulted in a decrease in u-PA mRNA content; however, this decrease (track 4, to 75% of control) was not as pronounced as that observed in the absence of cycloheximide (tracks 2 and 6, 30–50% of control in different experiments).

In the presence of actinomycin D, the level of u-PA mRNA
Glucocorticoid Regulation of Plasminogen Activators

FIG. 6. Time course of dexamethasone effect on u-PA and t-PA mRNA content in HBL-100 cells. Total RNA from confluent HBL-100 cultures maintained in DMEM or DMEM containing 10^{-7} M dexamethasone was prepared at different times. The RNA was dotted on nitrocellulose paper and hybridized to u-PA [32P]c-RNA (A) or t-PA [32P]c-RNA (B) probes. The dots were excised and counted. The values represent the mean ± SE of three separate determinations.

FIG. 7. Effects of protein and RNA synthesis inhibitors on glucocorticoid regulation of u-PA mRNA. HBL-100 cells were incubated for 7 h in DMEM (tracks 1 and 5), DMEM supplemented with 10^{-7} M dexamethasone (dex, tracks 2 and 6), 10 μg/ml of cycloheximide (cyc., track 3), cycloheximide plus dexamethasone (track 4, dexamethasone was added after 30 min of exposure to cycloheximide), 5 μg/ml of actinomycin D (act., track 7), actinomycin D plus cycloheximide (track 8, both drugs were added at the same time). Total cellular RNA was extracted from these cultures as well as from t-PA-producing Bowes melanoma cells (track 9) and u-PA-producing Hep-3 epidermoid carcinoma cells (track 10). Northern blot hybridization was performed using 12 μg of RNA/track. The filter was hybridized to a u-PA [32P]c-RNA probe which hybridized to RNA from Hep-3 cells, but not to RNA from Bowes melanoma cells. Quantitative analysis of the corresponding samples was done by dot-blot hybridization. The results represented in the bar graphs are the mean ± SE of three separate determinations.

FIG. 8. Effects of protein and RNA synthesis inhibitors on glucocorticoid regulation of t-PA mRNA. Analysis of total cellular RNA was done by Northern blot (a second hybridization of the blot shown in Fig. 7) and by dot-blot hybridization using a t-PA [32P]c-RNA probe. As expected, this probe hybridized to Bowes melanoma cells RNA but not to Hep-3 cells RNA. For further explanations of the legend and methods used see the legend to Fig. 7.

The present study of the plasminogen-dependent proteolytic system of HBL-100 cells has revealed a complex pattern that involved the production of both types of PA and of two distinct PA-specific inhibitors. Furthermore, glucocorticoid modulation of this system affected in opposite ways the production of the two PAS. An accurate description of this system must thus involve a variety of experimental approaches. Overall plasminogen-dependent proteolytic activity in cell extracts and culture media was first estimated by a caseinolytic assay. This procedure did not distinguish between u-PA and t-PA activity; in addition, since trace amounts of plasmin were probably present in our assay, this test did not distinguish between u-PA and its inactive proenzyme. The zymographic method proved very useful to resolve u-PA- and t-
Glucocorticoid Regulation of Plasminogen Activators

PA-related activities in samples from cultures of HBL-100 cells. For the reason mentioned above, zymography did not distinguish between the proenzyme and active u-PA; however, incubation of the samples with diisopropyl fluorophosphate (which does not react with one-chain pro-u-PA (46)), prior to SDS-PAGE and zymography, indicated that most of the u-PA in HBL-100 cells and culture medium was present as a diisopropyl fluorophosphate-resistant proenzyme (not shown). Zymography also revealed the catalytic activity of complexes between PAAs and their inhibitors; these complexes were characterized with the help of antibodies directed against the different enzymes and inhibitors. Taken together, these methods provided a reasonably quantitative evaluation of the different PA species in these cultures; however, we cannot rule out that part of the free t-PA resulted from dissociation of t-PA-inhibitor complexes during analysis of the samples. Despite this limitation, we concluded that control HBL-100 cells contained and secreted nearly exclusively pro-u-PA; upon exposure to dexamethasone, u-PA-related activity was markedly decreased, and two t-PA-related forms, i.e. free enzyme and a t-PA-inhibitor complex, were recovered from the culture medium. It is not known whether formation of the t-PA-inhibitor complex occurred in the cell, as has been observed in bovine endothelial (23) and rat hepatoma (27) cells, or whether the enzyme was secreted and then reacted with the inhibitor in the medium. These results pointed toward a complex pattern of glucocorticoid effects, and additional approaches, not involving the catalytic activities of the enzymes, were required.

In HTC rat hepatoma cells, glucocorticoids inhibit PA activity by inducing the synthesis of a PA inhibitor (27). To detect free PA inhibitors we used two different techniques which yielded apparently conflicting results. After addition of $1^{125}$-u-PA, the autoradiographic assay revealed the presence of free, unreacted, placental-type inhibitor. In contrast, reverse autography detected endothelial-type inhibitor. In addition to free PA inhibitors, we also identified in the culture medium a complex formed between endothelial-type inhibitor and t-PA. Hence, there are probably two pools of endothelial-type inhibitor in HBL-100 cell cultures: one which is almost entirely complexed to t-PA, and the other, a latent unreacted form, which requires activation by SDS (47). Furthermore, placental-type inhibitor is also present in these cultures; its activity was not revealed by reverse autography, probably because it was irreversibly denatured upon exposure to SDS.

A quantitative estimation of the autoradiographic assay indicated that the free placental-type inhibitor was present in large excess (10-100-fold) over PAAs. The coexistence in culture media of both placental-type inhibitor and u-PA, in its inactive proenzyme form, has already been reported (24, 26). Simultaneous detection of free t-PA and placental-type inhibitor can be explained in different ways: first, as discussed above, there may be no free t-PA in the culture medium, and its detection by zymography may result from dissociation of the complex during analysis of the samples; alternatively one-chain t-PA may not react with the placental-type inhibitor, as previously suggested (48).

Glucocorticoid treatment of HBL-100 cells did not detectably affect the accumulation of free placental-type inhibitor, nor that of free endothelial-type inhibitor. Since in HBL-100 cells the molecular mechanisms responsible for the glucocorticoid-induced changes in PA activity did not appear to involve changes in inhibitor production, an analysis of the synthetic potential for the enzymes themselves was thus required. The recently described use of single-stranded cRNA probes provides a highly sensitive and quantitative assay for mRNA levels (37). Culture of HBL-100 cells in the presence of dexamethasone resulted in a concomitant decrease in u-PA and increase in t-PA mRNA levels; the magnitude of both effects was similar. Further experiments will be necessary to establish whether these changes reflect altered transcription of the PA genes, altered mRNA turnover, or both. Experiments with inhibitors of macromolecular synthesis showed that the glucocorticoid-induced increase in t-PA mRNA required ongoing transcription but was unaffected by inhibition of protein synthesis. In contrast, the steroid effect on u-PA mRNA was not observed in absence of macromolecular synthesis, suggesting that it is a secondary event requiring the synthesis of regulatory protein(s). This is reminiscent of the effect of dexamethasone on PA production by macrophages (19): in this case also, a decrease in enzyme level was prevented by actinomycin D (see also Refs. 13, 15, and 16); it is now established that monocytes-macrophages produce the u-PA type enzyme (26) and that treatment of these cells with dexamethasone markedly decreases u-PA mRNA levels.

It appears doubtful that the observed changes in PA mRNA levels are sufficient to account for the effect of glucocorticoids on overall PA activity; for instance, accumulation of u-PA was decreased up to 10-fold in presence of dexamethasone, whereas u-PA mRNA content was decreased only 2-fold. Similarly, the 2-fold increased t-PA mRNA content appears insufficient to account for the marked increase in t-PA-related enzyme activity. Several factors may account for such discrepancies (49): the translational efficiency of PA mRNAs may vary, or PA accumulation may be influenced by clearance mechanisms, possibly involving the PA inhibitors (50, 51). In any event, the modulation of u-PA and t-PA mRNA levels in HBL-100 cells provides a potentially useful system to study at the molecular level both direct effects of glucocorticoids on gene expression and secondary events that involve the synthesis of regulatory proteins.

Acknowledgments—We thank N. Gerber for photographic work and G. Marchi for help in preparing the illustrations.

REFERENCES


M. Collart, D. Belin, J. D. Vassalli, and P. Vassalli, manuscript in preparation.
Glucocorticoid Regulation of Plasminogen Activators

Material and Methods

Preparation of plasminogen activators and their inhibitors in a human mammary cell line (HBL-100): Modulation by glucocorticoids by Nathalie Basso, Dominique Belin, Christine Fally-Griffith, and Jean-Michel Vassalli. The cells were grown in monolayer in DMEM/F12 supplemented with 10% FBS, 1% of a 1000-fold excess of unlabelled ligand. The final absorbance at 600 nm was plotted as a function of incubation time. The plasminogen-dependent activity was calculated as the difference between the absorbance obtained with urokinase standards, and was expressed as Ploug Unit/mg Cell Protein.

Cell Electrophoresis and zymography of Plasminogen activators (PA) were performed as previously described (26). Molecular weights were calculated from the positions of markers electrophoresed in the same gel lane and stained with Coomassie Blue. The molecular weight of human u-PA, t-PA, and m-PA was determined by the method of Granelli-Piperno and Reich (33) as modified by Vassalli et al. (26).

Assay for PA inhibitor (31). Labelling of u-PA, complex formation with PA inhibitors and autoradiography were performed exactly as described (28). Reverse autoradiography (32) was performed on the same gel used for zymography. The PA inhibitor was incubated with 10 ng of u-PA in the gel used for zymography. The complexes were electrophoresed for 3.5 hours at 150 V, and the gel was exposed to x-ray film at -80°C. The absorbance at 600 nm was plotted as a function of the length of the chain. The cell extract was 0.8 - 1 mg/ml as measured by the method of Bradford (31) using BSA as a standard.

Sensitivity assays with 10 ng u-PA and 10 ng u-PA were carried out on Dr. K. S. Schlegel (34,35,36). Tissue culture supernatant and 3-H MNC were used as substrates. The samples were centrifuged for 10 min at 500 X g and 5°C. The concentrations of plasminogen activators and their inhibitors were determined by gel zymography (26).

Preparation of culture media and cell extracts: Cultures were seeded at a density of 2 × 10^6 cells/ml in 96-well microtest plates at 37°C with 100 ml of DMEM-F12 without phenol red, 10% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 4 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES buffer (pH 7.4). The media were changed every 2 days and collected in 1 ml of a 1000-fold excess of unlabelled ligand. The final absorbance at 600 nm was plotted as a function of incubation time. The Ploug Unit/mg Cell Protein activity was calculated as the difference between the absorbance obtained with urokinase standards, and was expressed as Ploug Unit/mg Cell Protein.

Sample preparation: The samples were centrifuged for 10 min at 500 X g and 4°C. The cell extracts were used in gel zymography at 100 X g and 4°C. The final absorbance at 600 nm was plotted as a function of incubation time. The cell extract was 0.8 - 1 mg/ml as measured by the method of Bradford (31) using BSA as a standard.
were digested with 1-2 units of RNase-free DNase for 20 min at 37°C. After the addition of 20 μl of 1% SDS, 10 mM EDTA, 2 mg/ml tRNA and two extractions with phenol/chloroform, the RNAs were purified by Sephadex G50 chromatography in 10 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA and ethanol precipitation.

Northern-blot hybridizations - RNAs were denatured with glyoxal, electrophoresed in 1.2% agarose gel and transferred overnight onto nylon membranes according to Thomas (39). Filters were baked 2 h at 80°C and boiled for 5 min in 20 mM Tris-HCl pH 8.1 to remove residual glyoxal. Filters were prehybridized for 3 h at 58°C with 200 μl/cm² of hybridization mixture, containing 5 ng/ml of 32P-rRNA probe. The hybridization mixtures contained 50% (v/v) denatured formaldehyde, 50 mM Na-Pipes pH 6.8, 0.1% SDS, 0.2% salmon sperm DNA. The mixtures were pre-heated, filtered through nitrocellulose and degassed before use. Filters were washed twice at 58°C with 2 x SSC, 0.1% SDS, and 0.1% Na-Pyrophosphate. Finally, the filters were exposed to Kodak XAR-5 films at -80°C between intensifying screens. After a first hybridization, the probe was eluted from the filters by boiling for 5 min in 20 mM Tris-HCl pH 8.1. Filters were exposed to ensure complete removal of the probe and subsequently subjected to a second hybridization with the other probe.

Dot-blot hybridization - RNAs were denatured with formaldehyde (40), serially diluted with 15 x SSC, 0.1% SDS, 0.1% RNase and Schleicher & Schuell mini-fold apparatus for dot hybridization. Filters were baked, prehybridized, washed, and autoradiographed for a visual evaluation of relative mRNA levels as described above. Quantitative results were obtained by cutting the dots and counting them by liquid scintillation.

RESULTS

Binding of dexamethasone to HBL-100 cells

HBL-100 cells are a suitable in vitro model system for studying glucocorticoid regulation, since the only steroid receptor expressed in these cells is that for glucocorticoids (41). We have determined the binding parameters of dexamethasone, a synthetic glucocorticoid, to HBL-100 cells. Specific binding reached saturation at about 10⁻⁸ M (μg) dexamethasone (Figure 1A). The Scatchard analysis of the data (Figure 1B) suggests the presence of a single class of receptor sites of high affinity. In two separate experiments we calculated that there were close to 30'000 sites per cell with a Kd of 4.10⁻⁹ M.