Lack of gradual regulation of tetracycline-controlled gene expression by the tetracyclin-repressor/VP16 transactivator (tTA) in cultured cells

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Abstract Von Willebrand factor (vWF) is an essential multimeric protein for adhesion of platelets to an injured vessel wall. Endothelial cells secrete vWF by either a constitutive or a regulated pathway. It is unknown whether the secretory partitioning of vWF is dependent on the level of vWF synthesis. We employed the widely applied tetracycline-controlled transactivator system (tTA) to study the regulation of vWF mRNA synthesis in stably transfected Madin Darby kidney (MDCK-II) cells in a quantitative manner. Immunofluorescence staining with anti-vWF antibodies revealed that increasing the concentration of tetracycline resulted in a decreased number of MDCK-II cells that synthesize vWF. Apparently, tTA-regulated gene expression in an individual cell functions as an 'on/off' system rather than regulating the level of gene expression in a dose-response manner, as reported previously.

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1. Introduction

Von Willebrand factor (vWF) is a large multimeric glycoprotein that plays an essential role in hemostasis. It primarily acts as a 'molecular bridge' that connects the platelet to the subendothelium, exposed upon injury of the vessel wall. The biosynthesis of vWF is restricted to two cell types, i.e. the endothelial cell and the megakaryocyte. In the endothelial cell, vWF that exits the trans-Golgi network is routed by two different pathways, denoted the constitutive and the regulated pathway (reviewed in [1]). Secretion by the latter pathway is typified by storage of vWF in endothelial cell-specific organelles (Weibel-Palade bodies) and release upon activation with an appropriate agonist. Constitutively secreted vWF consists of dimers and low molecular weight multimers, whereas vWF released from the Weibel-Palade bodies is contained in high molecular weight multimers. The latter species are particularly effective ligands for platelet adhesion [2]. At present, factors or conditions that determine the secretory partitioning of vWF (at different levels of expression) are unknown, although it has been hypothesized that for the secretion of hormones the constitutive route may represent a default pathway, operative when the regulated pathway is 'saturated' [3].

To study this issue, we used a system that is widely employed to regulate protein synthesis in cultured cells and in transgenic mice by varying the corresponding mRNA synthesis [4]. This regulatory system exploits the sensitivity of mammalian cells for the bacterial antibiotic tetracycline (tet) as

well as their ability to functionally synthesize the prokaryotic tet repressor. The tet repressor has been converted into a eukaryotic transactivator by fusion with the activator domain of virion protein 16 (VP16) of the Herpes Simplex virus type 1 (denoted the tet-controlled transactivator or tTA). This protein stimulates transcription initiated at a promoter, derived from human cytomegalovirus (CMV). The enhancer region of this promoter has been removed and substituted by eight consecutive tet operator sequences. In the absence of tTA, this 'minimal promoter' is virtually inactive. HeLa cells, which constitutively express tTA and are stably transfected with DNA encoding luciferase, preceded by the minimal CMV promoter, synthesize a high level of luciferase. The stimulation of luciferase expression by tTA can be gradually reduced by administration of tet which interferes with the binding of tTA to the tet operators. It is assumed that tTA provides for a tightly controlled gene expression system in which the level of expression in each cell is a direct consequence of the tet concentration [4].

Here, we employed the tTA system to regulate vWF synthesis in Madin Darby kidney cells that had been stably transfected with vWF cDNA and that constitutively express tTA (MDCK-II/vWF). MDCK-II cells lack endogenous vWF expression, but are able to secrete vWF both by a constitutive and by a regulated pathway upon transfection with vWF cDNA [5]. The data confirm that the tet concentration determines the level of vWF expression of a population of cells as has been reported before [4]. However, inspection of individual cells by immunofluorescence demonstrated that the level of vWF synthesis of the cell population is a reflection of the number of cells that produce vWF rather than the resultant of an identical, defined level of expression of each individual cell.

2. Materials and methods

2.1. Plasmids

Plasmids pUHD15-1 (containing the tTA gene), pUHD13-3 (containing the minimal promoter and the luciferase reporter gene) and pUHD10-3 (containing the minimal promoter and a multiple cloning site) were kindly provided by Dr. H. Bujard (Center of Molecular Biology, University of Heidelberg, Heidelberg, Germany). Plasmid p-hd (containing the hygromycin resistance gene) was kindly provided by Dr. B. Grinnell (Lilly Corp., Indianapolis, IN, USA). VWF cDNA, obtained by digestion of pSVL-vWF DNA [6] with *Eco*RI, was ligated into *Eco*RI-digested pUHD10-3 DNA, yielding pUHD10-3-vWF.

2.2. Tissue culture

MDCK-II cells were cultured in Iscove's modification of Eagle's medium, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (0.25 µg/ml) and 10% (v/v) fetal calf serum (FCS). The cells were split twice a week (surface dilution 1:4) using trypsin to suspend the cells. Cells were kept at 37°C in a moist atmosphere in a 5% CO_2/95% air incubator.

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2.3. Luciferase assay

Cell extracts were prepared and assayed for luciferase activity as described [7] using a Luminat LB 9501 illuminometer (Berthold).

2.4. Immunofluorescence

The procedure to visualize vWF protein, using a 1:500 dilution of rabbit polyclonal anti-vWF serum (Dakopatts) as the first antibody and a 1:300 dilution of Cy-3-conjugated goat anti-rabbit antiserum (Jackson Immuno Research Lab.) as the second antibody, has been described before [6]. The cells were viewed with an Olympus IMT2 fluorescence microscope.

2.5. RNA isolation and Northern blotting

Total RNA of various cell lines was isolated with Trizol reagent, according to the manufacturer's instructions (Gibco Life Technologies Inc.). Northern blotting of 10 µg of RNA was essentially done as described [8]. A 490 bp BamHI-HindIII vWF cDNA fragment, radio-labelled with the random primer DNA labeling system (Gibco Life Technologies Inc.) and α-[^{32}P]dATP, was used as a probe for RNA:DNA hybridization. Non-incorporated radioactivity was removed by Sephadex G-50 chromatography. After hybridization, radioactive probes were removed by incubating the blots twice for 20 min at 90°C in 0.1×SSC, 0.1% (w/v) SDS, 1% (w/v) sodium pyrophosphate. Subsequently, the blots were reutilized for hybridization with a radiolabelled 89 bp EcoRI-SalI cDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for calibration of the amount of RNA applied.

3. Results

3.1. Stable integration of pUHD15-1 in MDCK-II cells

To obtain MDCK-II cells that constitutively express tTA, we cotransfected pSV2neo (carrying the neomycin resistance (neo^R) gene) with a molar excess of pUHD15-1 DNA (encoding tTA) [9]. Thirty neo^R clones were isolated of which 22 survived prolonged growth in a G418-containing medium. To verify whether these clones contain stably integrated tTA cDNA that encodes functionally active tTA protein, a transient transfection with pUHD13-3 was performed. This plasmid contains DNA, encoding luciferase, coupled to the minimal CMV promoter. Subsequently, luciferase activity was measured in extracts of neo^R cells. Nine extracts displayed luciferase levels that were 7–40-fold higher than untransfected MDCK-II cells (Fig. 1). In addition, the same experiment was

Fig. 2. Luciferase activity of selected MDCK-II-tTA cell lines after transient transfection with pUHD13.3 cultured at different tet concentrations. Luciferase activity was measured in extracts of the MDCK-II-tTA cell lines, MDCK-II-V25, MDCK-II-V20 and MDCK-II-V9, and in extracts of control MDCK-II cells (C) that were each transiently transfected with pUHD13.3, containing DNA encoding luciferase that is preceded by the minimal CMV promoter. RLU: relative light unit.

performed in the presence of a high concentration of tet (1 µg/ ml). At this concentration of the antibiotic, the luciferase synthesis was reduced in extracts of each clone. However, a reduction of luciferase activity in the presence of 1 µg/ml tet to background levels of untransfected cells was observed in only three cases. These three clones, denoted MDCK-II-V9, MDCK-II-V20 and MDCK-II-V25, were selected for further analysis. A similar transient transfection experiment, at various tet concentrations, showed that clone MDCK-II-V25 displays the highest activity in the absence of tet, whereas under these conditions MDCK-II-V20 produced approximately half the amount of luciferase per cell and MDCK-II-V9 about 25%, relative to MDCK-II-V25 (Fig. 2). Clearly, in the presence of 0.1 µg/ml tet the expression of luciferase in each of these clones is reduced to background levels.

3.2. Stable integration of pUHD10-3-vWF in MDCK-II-V25

MDCK-II-V25 cells were used for the stable integration of vWF cDNA, preceded by the minimal CMV promoter. A cotransfection was performed with p-hd (carrying the hygromycin resistance gene (hyg^R)) and an excess of plasmid pUHD10-3-vWF [9]. Twenty hyg^R clones were isolated and 14 survived prolonged growth in selective medium. Analysis of vWF expression was performed by immunofluorescence, revealing that eight clones displayed both perinuclear vWF staining and a punctate fluorescence pattern, representing storage organelles as shown before [5]. Two clones that display the most extensive vWF staining, denoted MDCK-II-V25.59 and MDCK-II-V25.66, were selected for further analysis.

3.3. Analysis of vWF mRNA levels at different tet concentrations

The amount of vWF mRNA levels of MDCK-II-V25.59 cells, cultured at different tet concentrations, was determined by Northern blotting and phosphorimager analysis. To that end, total RNA was isolated from cells cultured grown until confluency in the presence of the indicated tet concentrations
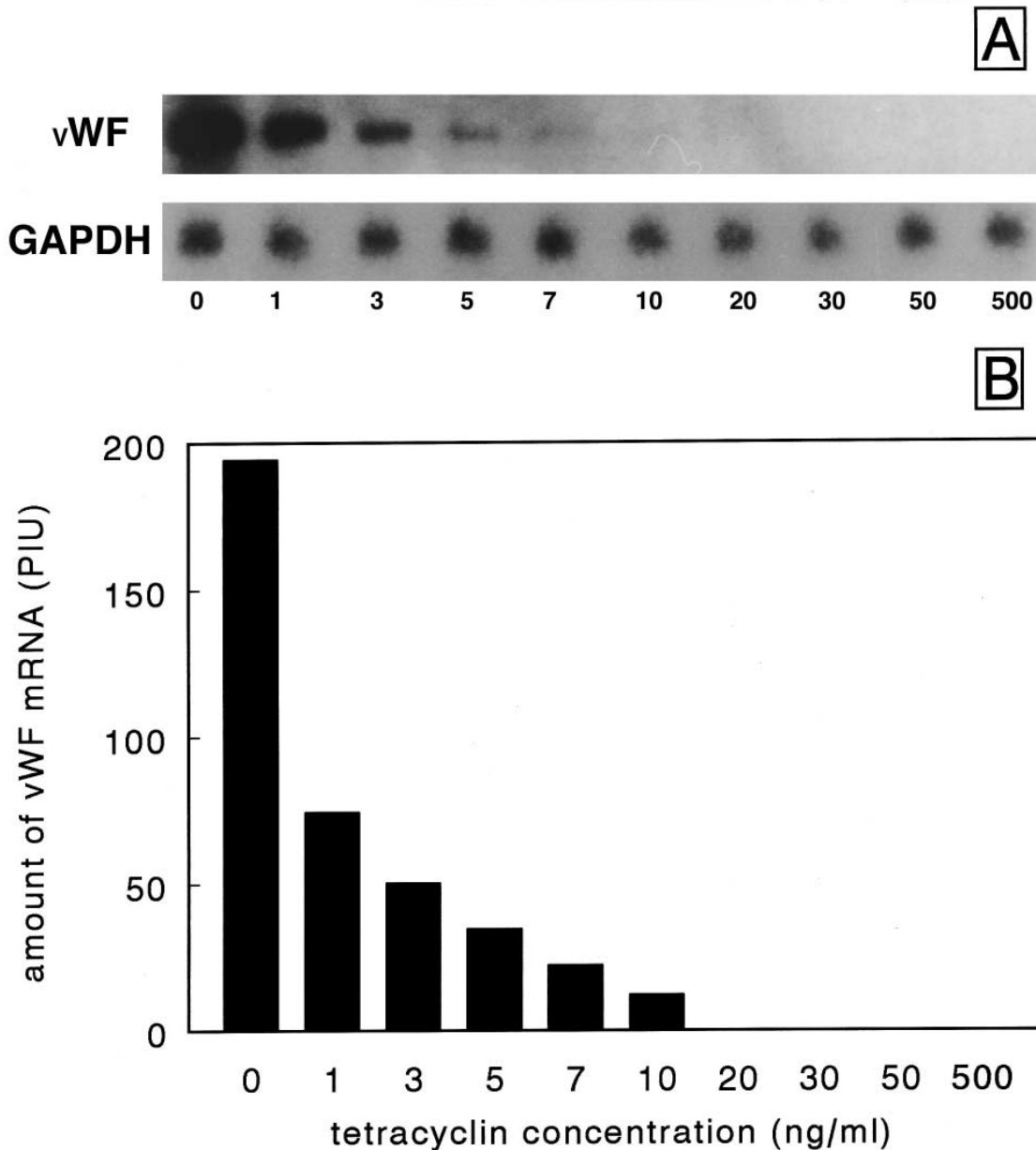
Fig. 1. Luciferase activity of various MDCK-II-tTA cell lines after transient transfection with plasmid pUHD13.3. Luciferase activity was measured in extracts of various MDCK-II-tTA cell lines and control MDCK-II cells (C) that were transiently transfected with pUHD13.3 DNA, containing DNA encoding luciferase that is preceded by the minimal CMV promoter. The cells were cultured in the absence (−) or presence (+) of 1 µg/ml tet. RLU: relative light unit.

Fig. 3. Northern blotting of RNA isolated from cell line MDCK-II-V25.59, cultured at different tet concentrations. A: Lanes 1–10: Autoradio-gram of the Northern blot of MDCK-II-V25.59 RNA, cultured at the indicated tet concentrations. A 490 bp fragment of vWFcDNA was used as a probe. B: Results obtained by phosphorimaging of the bands shown in (A), corrected for the differences in RNA amounts as determined by probing with a 89 bp fragment of GAPDH cDNA. PIU: phosphorimager unit.

(Fig. 3). Clearly, the amount of vWF mRNA decreases upon increasing tet concentration and has completely disappeared in the presence of 20 ng tet/ml. By employing a vWF antigen-specific ELISA, we obtained virtually identical results for vWF protein synthesis as a function of different tet concentrations (data not shown).

3.4. Immunofluorescence analysis of vWF expression by MDCK-II-V25.59 and MDCK-II-V25.66 cells

The experiments described above allow two interpretations: (1) each individual cell expresses vWF at a level corresponding to the tet concentration, (2) the tet concentration determines the number of cells participating in vWF synthesis. To discriminate between these two options, we inspected individual

cells by immunofluorescence to analyze vWF expression by cultured MDCK-II-V25.59 and MDCK-II-V25.66 cells at different tet concentrations (Fig. 4A). Furthermore, the number of immunofluorescent cells was precisely counted and plotted against the tet concentration employed (Fig. 4B). The data clearly show that the number of immunofluorescent cells is reduced upon increasing the tet concentration. To analyze the correlation between the number of immunofluorescent cells at a particular tet concentration and the amount of vWF mRNA, the data on the amount vWF mRNA synthesized as a function of the tet concentration (see Fig. 3) were combined with those obtained on the number of immuno-fluorescent cells at the same tet concentration (Fig. 5). This figure demonstrates a precise correlation between the number
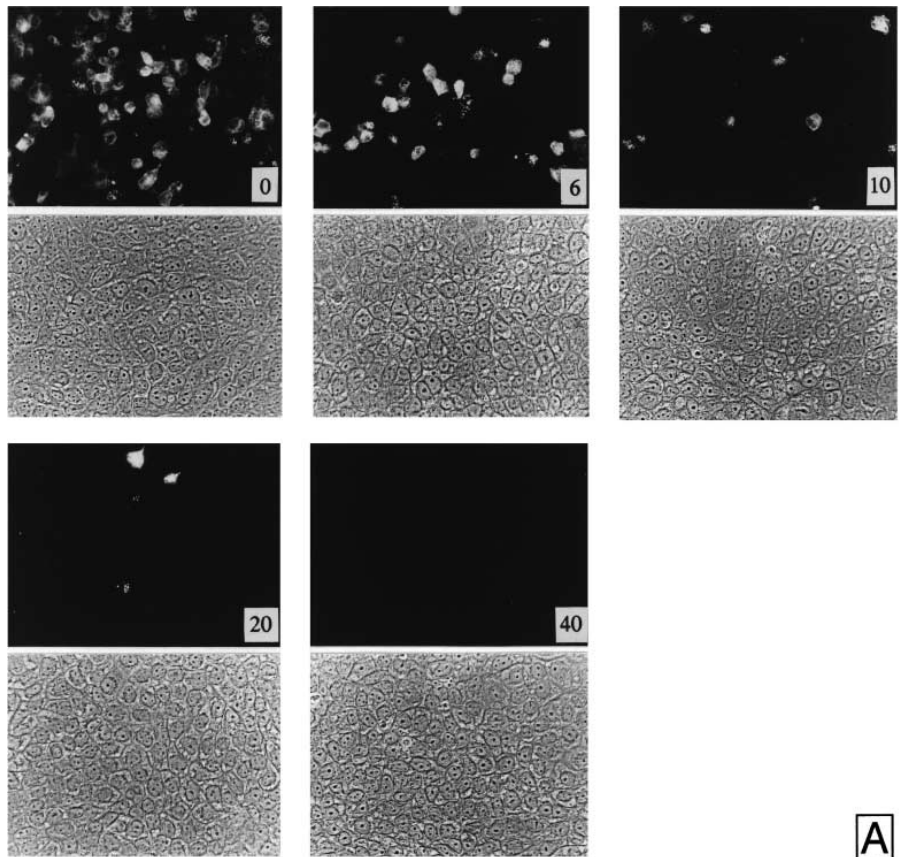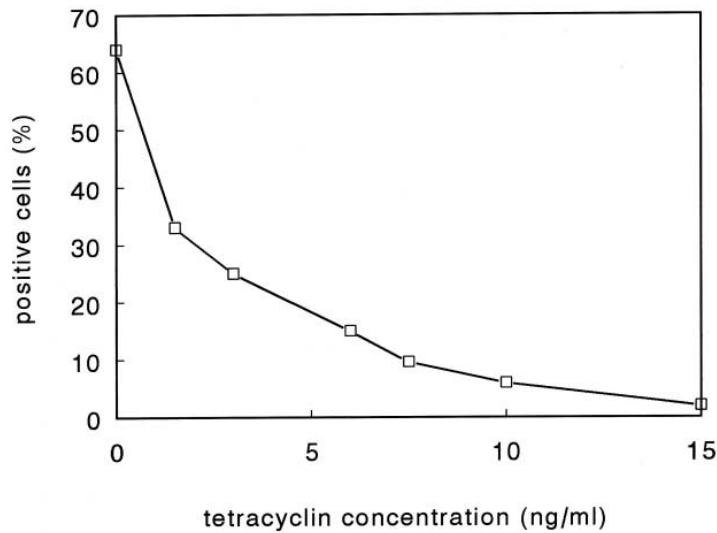
Fig. 4. The number of fluorescent MDCK-II-V25.59 cells, cultured at various tet concentrations. MDCK-II-25.59 cells were grown on cover-slips until confluency at the indicated tet concentrations. A: Immunofluorescence for vWF and phase contrast analysis of different sectors of cells. B: The percentage of vWF-expressing cells per sector was determined and plotted against the tet concentration.

of cells that display vWF expression and the amount of vWF mRNA that is synthesized at different tet concentrations.

4. Discussion

In the present study, we attempted to exploit the tetracy-cline-controlled transactivator (tTA) system to address the following issue: is the distribution of vWF between the two distinct secretory pathways affected by its level of expression? Obviously, we did not resolve this issue due to unexpected findings on the mechanism of the widely employed tTA sys-tem to regulate gene expression. Our data actually contradict the view that regulation of gene expression by the transacti-vator tTA in an individual cell occurs in a gradual mode that is dependent on the tet concentration employed. The original concept assumes that the level of tTA-dependent gene expres-

MDCK-II cells is susceptible to tet, possibly due to a particularly sensitive phase in the cell cycle. In that case, the sensitivity of other phases of the cell cycle apparently requires a higher dose of the antibiotic.

The significance of the tetracycline-controlled transactivator system clearly goes beyond studies to regulate gene expression in cultured cells. At present, the tTA system is widely employed to study temporal regulation of gene expression in transgenic mice by either providing or withdrawing tet from the diet of these animals [11–13]. An extrapolation of the data reported in this study to the effect of decreasing concentrations of tet in the diet of transgenic mice, having a transgene under the control of tTA, would imply that an increasing number of cells would participate in the synthesis of the transgenic protein rather than a gradually increased mRNA and protein synthesis. The ultimate result for proteins delivered to the bloodstream would be similar, irrespective of the mechanism by which tet controls tTA-driven synthesis. However, in the case of an intracellular transgene under the control of tTA our data would predict a mosaic expression pattern dependent on the concentration of the antibiotic supplied.

Fig. 5. Comparison between the number of vWF-expressing cells and the vWF mRNA levels of MDCK-II-V25.59 cells, cultured at various tet concentrations. The cells were grown in 56 cm^2 Petri dishes until confluency in medium, provided with the indicated tet concentrations. RNA was isolated and used for Northern blotting as described in Section 2. MDCK-II-25.59 cells were grown on coverslips until confluency at the indicated tet concentrations. Immunofluorescence was performed and the number of fluorescent cells was counted per field. Both values, from the Northern blotting as well as the counted fluorescent cells, were plotted against the tet concentration as a percentage of the values in the absence of the antibiotic.

sion is dependent on the tet concentration, administered to the cells [4]. However, our data obtained with MDCK-II cells that constitutively express tTA and that have been stably transfected with vWF cDNA, provided with the minimal CMV promoter, indicate that tTA-driven gene expression in a cultured cell is either fully operational (in the absence of tet) or completely repressed (in the presence of a defined threshold concentration of tet). Hence, under these conditions a gradual variation of the tet concentration does not allow for concomitant variation of the level of gene expression per cell. Analysis of a population of MDCK-II/vWF cells by immunofluorescence rather shows that administration of an increasing concentration of tet results in a decreased number of cells that participate in tTA-driven vWF synthesis. At present, a rationale for these observations is not readily available. It has been argued that the entry of the antibiotic into mammalian cells would be only dependent on passive diffusion through the cell membrane, in contrast to the entry in bacteria that occurs both by an active energy-dependent transport system and by passive diffusion [10]. If passive diffusion of tet into mammalian cells were the only mode of entrance then we assume that at low tet concentrations only a small fraction of these clonal

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