

FIG. 2. Time course of dimerization of Cit1p in a homogenous 10% BN-PAGE. (A) Radiolabeled Cit1p was imported into isolated mitochondria at 25°C for 7 min. The import reaction was stopped by addition of valinomycin. The incubation at 25°C was continued and samples were put on ice after the time points indicated. The mitochondria were reisolated by centrifugation and the mitochondrial proteins were separated by BN-PAGE. (B) Time course of dimerization of Cit1p in mitochondria after inactivation of Tim44 (in mitochondria isolated from the mutant *tim44-8*) and dimerization in wild-type mitochondria (WT). The results were quantified using a phosphorimager. The ratios of dimeric Cit1p to total (dimeric plus monomeric) Cit1p are shown. Standard deviations were calculated from three independent experiments.

Fig. 2A. Using a phosphorimager, we determined the relative amounts of dimers and monomers in wild-type and mutant mitochondria at different time points (Fig. 2B). The formation of Cit1p dimers was clearly delayed in the *tim44-8* mutant mitochondria compared to the wild-type mitochondria. Essentially the same results were obtained by exposure of X-ray films and subsequent densitometry. The variations of the values shown in Fig. 2B were mainly due to differences in the properties of individual preparations of mitochondria.

We conclude that homogenous 10% acrylamide blue native gels are suitable to characterize the dimerization of newly imported citrate synthase in mitochondria. It is

of advantage that no chemical modification of the Cit1p is necessary to investigate the time course and the specific requirements of dimerization *in organello*. Using mitochondria from mutants of different genes, it should now be possible to determine the components of the mitochondrial matrix that interfere with the dimerization. Moreover, we suggest that it may be worth trying to establish similar assays for other soluble proteins, although the initial purpose of the BN-PAGE system was the analysis of membrane proteins. In many cases it should be sufficient to use gels of homogenous acrylamide concentration and to omit the preparation of gradient gels.

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Unsuitability of Using Ribosomal RNA as Loading Control for Northern Blot Analyses Related to the Imbalance between Messenger and Ribosomal RNA Content in Rat Mammary Tumors

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Overexpression is a common genetic alteration in malignant tissues and therefore examining the expression of particular genes is frequent in biological studies

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of cell proliferation and cancer. Northern blot analysis is the most common method for analyzing messenger RNA (mRNA) gene expression levels and establishing quantitative comparisons among different sample types. There are, however, several methodological problems such as uneven loading or different efficiency of transfer between lanes that may cause errors when verifying changes in gene expression. One of the most popular approaches to solving them is the normalization of the gene of interest to a housekeeping gene, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin, cyclophilin, albumin, or acidic ribosomal phosphoprotein genes, that is constitutively expressed in each cell or tissue independently of experimental conditions. The fact that steady-state levels of these control genes cannot always be assumed has led to the development of an alternate approach based on the ribosomal RNA (rRNA) (1, 2). It is supposed that more than 90% of the total RNA is rRNA and its level appears to be a constant fraction of total RNA. Consequently, normalization of the amounts of RNA loaded by means of fluorescence staining of rRNA or by hybridization with a 28S- or 18S-specific antisense probes would represent a consistent method of verifying equal loading (3).

In our experience rRNA is not always a good internal control for Northern blot analyses. In this way, even though the UV image of rRNA shows the same amount of total RNA in all samples loaded in the Northern gel, occasionally in some of them the hybridization with specific probes corresponding to differentially expressed genes suggests that the messenger fraction could be decreased. Therefore, at least in these samples, the rRNA would not be representative of the mRNA and it could not be used to correct loading differences into the gel.

We have performed Northern blot analyses of total RNA from 121 mammary adenocarcinomas induced by 7,12-dimethylbenz(α)anthracene (DMBA)² in female Sprague-Dawley rats. We used specific probes for genes considered housekeeping (β -actin, GAPDH, and p0) and genes potentially implicated in mammary tumor development (c-Ha-ras and c-erbB): (1) a 1039-bp mouse β -actin cDNA (ATCC, U.S.A.), (2) a 777-bp human GAPDH cDNA (ATCC, U.S.A.), (3) a 756-bp rat p0 cDNA, kindly provided by Dr. Y.-L. Chan, (4) a 701-bp v-Ha-ras (ONCOR, Spain), and (5) a 2-kb rat EGFR cDNA, kindly provided by Dr. H. S. Earp.

Briefly, 20 μ g of total RNA, as determined by absorbance at 260 nm, were extracted by guanidinium thiocyanate-phenol-chloroform method (4) from frozen tissues (snap freezing in liquid nitrogen and storage at -80°C). Samples were electrophoresed in formalde-

hyde-agarose gels and blotted onto positively charged nylon membranes (Roche Molecular Biochemicals, Germany) by alkaline transfer procedure. After UV crosslinking, blots were prehybridized in 0.25 M Na_2HPO_4 , pH 7.2, 1 mM EDTA, 20% (w/v) SDS, and 0.5% (w/v) blocking reagent (Roche Molecular Biochemicals) for 1 h at 65°C , and hybridized overnight at 65°C with PCR Digoxigenin-labeled DNA probes at 10–25 ng/ml. They were then washed three times for 20 min at 60°C with washing buffer (20 mM Na_2HPO_4 , pH 7.2, 1 mM EDTA, pH 8.0, 1% (w/v) SDS).

For the immunological detection, CSPD was used as the chemiluminescent substrate. Membranes were exposed to X-ray film from a few minutes to several hours. The X-ray films were digitized using a transmission scanner and densitometry of the scanned images was performed using the Gel Doc 2000 image analyzer system and the Quantity One software system (Bio-Rad, Spain). The capture and densitometry of the ethidium bromide-stained gel image were also performed with this system. The densitometric value obtained for c-Ha-ras and c-erbB mRNAs for each sample was normalized relative to β -actin, GAPDH, and p0 mRNAs, and to 28S rRNA.

Among the 121 mammary tumors analyzed we detected the above described imbalance between the rRNA and mRNA fractions in 9 samples (7.5%). In Fig. 1 we show a representative Northern blot with this kind of samples. Thus, tumors loaded in lanes 2, 4, 6, 8, and 10 have a marked reduction of the hybridization signal for all five genes tested (b, c, d, e, and f) despite the image of the ethidium bromide-stained gel (a) displayed an even loading in all of them (lanes 1 to 10). Therefore, if using the 28S rRNA to normalize c-Ha-ras and c-erbB mRNAs signals, it could be concluded that the tumors in lanes 2, 4, 6, 8, and 10 underexpress these genes relative to the tumors in lanes 1, 3, 5, 7, and 9. On the other hand, when normalizing to the "housekeeping" genes mRNAs, the result is different and, in addition, variable depending on the control used (see ratios on table, Fig. 1g).

The fact that the decrease of the intensity of the signal is obtained for both genes with a relatively high expression, such as actin, GAPDH, and p0, and for genes with a relatively low expression in these experimental tumors, such as c-Ha-ras and c-erbB, suggests that it would not be due to an underexpression, possibly associated to the tumor development, but to a more general phenomenon that would affect all the mRNA population. For example, a differential regulation of the RNA polymerases I and II, responsible of transcription of genes which codify rRNA and mRNA, respectively, could produce an rRNA/mRNA imbalance. In this way, it has been described that during rapid growth and differentiation the levels of rRNA may vary (5). In our mammary tumor samples, the levels of

² Abbreviation used: DMBA, 7,12-dimethylbenz(α)anthracene.

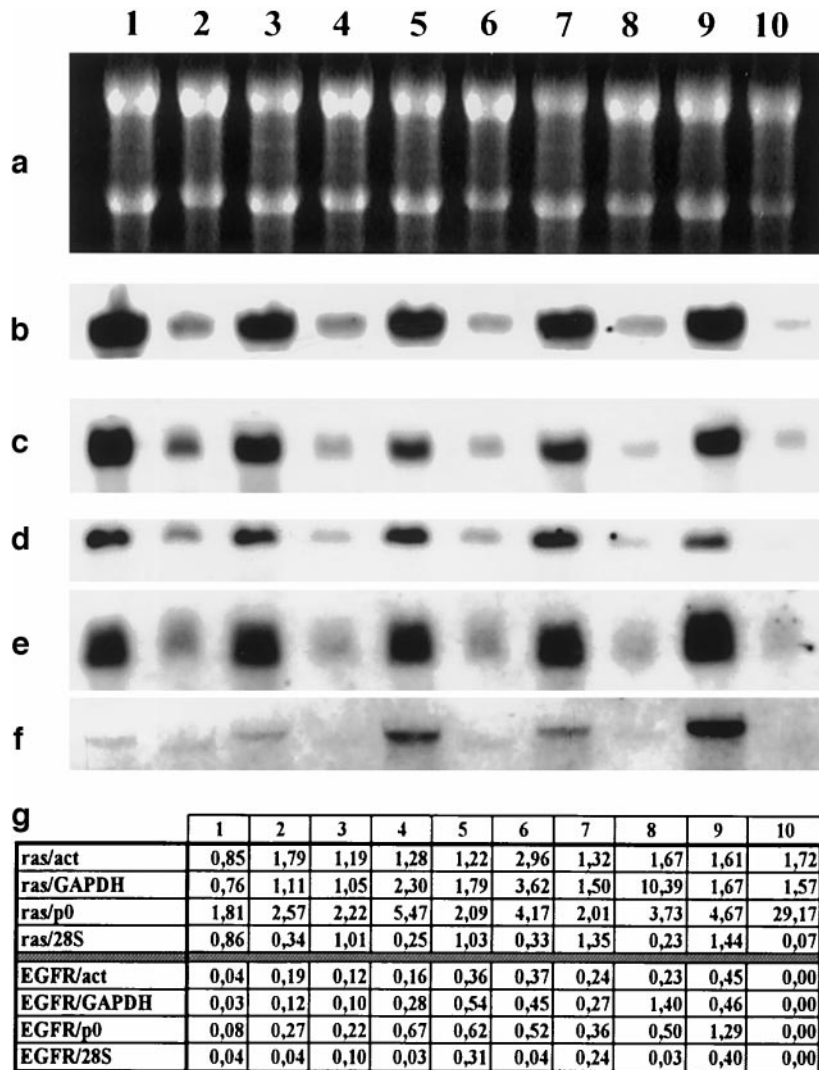


FIG. 1. Representative Northern blot of total RNA from rat mammary tumors (lanes 1 to 10). Lanes 2, 4, 6, 8, and 10: samples with the imbalance between the rRNA and mRNA fractions; lanes 1, 3, 5, 7, and 9: samples without this phenomenon. (a) Visualization of 28S and 18S rRNA by ethidium bromide staining of Northern gel. (b) 2.1-kb β -actin mRNA; 20-min exposure time. (c) 1.4-kb GAPDH mRNA; 1-h exposure time. (d) 1.1-kb ribosomal phosphoprotein p0 mRNA; 1 h 30 min exposure time. (e) 1.4-kb c-Ha-ras mRNA; 4-h exposure time. (f) 9-kb c-erbB mRNA; 6-h exposure time. (g) Normalization of the specific densitometric signals for c-Ha-ras and c-erbB mRNAs relative to β -actin, GAPDH, p0 mRNAs, and 28S rRNA.

rRNA and/or those of mRNA could have varied depending on cell differentiation degree. Another possibility is a differential degradation of the two RNA populations that could affect some samples more than others. This could be related to the high nuclease activity described in the DMBA-induced mammary tumors (6). Whatever it is the cause, the analysis of gene expression in the unbalanced tumor samples could not be performed using rRNA to normalize.

In summary, we present some experimental evidences for which rRNA cannot always be used for normalization of the amounts of RNA loaded in Northern gels. In past years, a new approach based on the de-

termination of relative poly(A) RNA content has been widely used (7, 8). However, this method also has some disadvantages such as not all mRNAs have a poly(A) tail, its length can vary in different samples, and mRNA stability can be very different. There is probably no easy universal method to standardize samples, at least cancer samples, for control mRNA levels. Maybe the best would be to determine empirically the housekeeping gene for each tissue and experimental condition or to estimate the relative mRNA content. In any case, samples suspected to display changes in gene expression should be repeated multiple times to confirm the result.

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Quantitation of Cellulose Acetate Phthalate in Biological Fluids as a Complex with Ruthenium Red

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Cellulose acetate phthalate (CAP²; Eastman Chemical Co., Kingsport, TN) is a cellulose derivative in which approximately one-half of the hydroxyl groups are acetylated and approximately one-fourth are esterified with one of the two acid groups of phthalic acid;

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² Abbreviations used: CAP, cellulose acetate phthalate; STD, sexually transmitted disease; RR, ruthenium red.

the other acid group is free. CAP is used as an enteric film coating material or as a matrix binder for tablets and capsules (1). A micronized form of CAP (Aquateric; containing 66 to 73% CAP, Poloxamer, and distilled acetylated monoglycerides; FMC Corp., Philadelphia, PA) is used for coating from aqueous solvents. Recently, CAP and Aquateric were shown to inactivate sexually transmitted disease (STD) pathogens, suggesting that CAP formulations are candidate microbicides to prevent the acquisition of STDs, including AIDS (2–4). Analytical methods are needed to determine the stability in different environments and the *in vivo* distribution of CAP. Such methods have not been previously reported. We developed an HPLC method to quantitate CAP in distinct formulations. This method could not be applied to complex biological fluids. Therefore, another method had to be developed.

Sulfated glycosaminoglycans (5) and calcium-binding proteins (6) can be quantitated as complexes with ruthenium red (RR). Endo-1,4- β -glucanases can be detected based on enzymatic release of dyed fragments from carboxymethylcellulose–RR complexes (7). It seemed possible that CAP would also form complexes

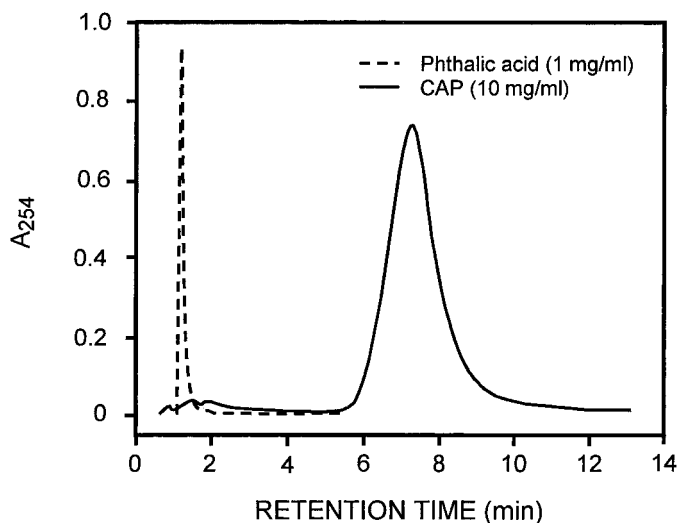


FIG. 1. Superimposed HPLC chromatograms of phthalic acid (1 mg/ml) and CAP (10 mg/ml). HPLC was performed in a Waters 600E multisolute delivery system with a Waters 996 photodiode array detector and Millennium 2010 chromatography manager with detection at 254 nm, using a VYDAC 301 VHP 575 column equilibrated with 20 mM borate, pH 8.5 (buffer A). Samples were diluted in A and when necessary, the pH was adjusted to 8.5, and centrifuged at 3000g for 5 min. Twenty microliters of the supernatants was applied to the column and subsequently eluted with a linear gradient (A – 1 M NaCl in A) at a flow rate of 1 ml/min. The method was used to determine the stability of CAP in NYB-122A suspended in 0.1 M borate, pH 8.8 (80 mg of formulation/ml). Due to hydrolysis, the integrated absorbance corresponding to the CAP peak decreased from 97.6% at time 0 to 65, 60, and 45% after 17, 20, and 30 h at 50°C, respectively, with a concomitant increase in the integrated absorbance corresponding to free phthalic acid.