Cell Size Regulation, a Mechanism That Controls Cellular RNA Accumulation: Consequences on Regulation of the Ubiquitous Transcription Factors Oct1 and NF-Y, and the Liver-enriched Transcription Factor DBP

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Abstract. Cell sizes can differ vastly between cell types in individual metazoan organisms. In rat liver, spleen, and thymus, differences in average cell size roughly reflect differences in RNA:DNA ratios. For example, hepatocytes were found to have a cytoplasmic:nuclear volume ratio and an RNA:DNA ratio which were 34- and 21-fold higher, respectively, than those in thymocytes. RNA synthesis per DNA-equivalent in the hepatocytes was 25-fold greater than that in thymocytes, suggesting that differences in overall transcriptional activity, not differences in overall RNA stability, were primarily responsible for determining cellular RNA:DNA ratios. The mechanisms determining the capacity of large cells to synthesize and accumulate more ubiquitous cytoplasmic macromolecules, such as ribosomes, than smaller cells is entitled "cell size regulation."

Cell size regulation may have important consequences on the tissue distribution of transcription factors. Thus, in liver, lung, kidney, spleen, and brain, cellular levels of the mRNA encoding the leucine zipper protein DBP correlate closely to cellular RNA:DNA ratios. Our results suggest that DBP mRNA levels, like rRNA levels, are transcriptionally determined. Thus the *dbp* gene, like the ribosomal genes, may be subject to cell size regulation. As a consequence, nuclei from liver, a tissue with a very large average cell size, accumulated higher levels of DBP protein than nuclei from small-celled tissues, such as spleen or lung. In contrast to DBP, the ubiquitous transcription factors Oct1 and NF-Y escaped cell size control. Nuclei from most tissues contained similar amounts of these factors irrespective of cell size. Likewise, tissues with large or small average cell sizes contained similar levels of the mRNAs encoding Oct1 or NF-Ya, one of the subunits of the heteromeric CCAAT-binding factor NF-Y, per DNA-equivalent. Interestingly, mRNA encoding NF-Yb, another subunit of NF-Y, was subject to cell size regulation. Our results suggest that NF-Yb protein escapes cell size regulation at a posttranslational level.

LATE in the 19th century biologists began investigating the relationship between nuclear and cytoplasmic content. In 1903, Hertwig hypothesized that the nuclear:cytoplasmic ratio is a natural constant. Indeed, if one compares a single cell type between haploid and diploid sea urchin embryos (Boveri, 1905), one finds that the diploid cells are twice as big as the haploid cells. Similar observations have been reported in comparisons between differentially polyploid protozoan, plant, or amphibian species (Artom, 1912; Fankhauser, 1945; Gerassimow, 1902; Seyfert et al., 1984; Wettstein, 1928), in comparisons of genome sizes to cell sizes between several hundred vertebrate species (Olmo, 1983), and even in comparisons of cell sizes between diploid and tetraploid mouse embryos (Henery and Kaufman, 1992). Clearly, a relationship between nuclear content and cell size exists. However, even as the studies above provide observations consistent with Hertwig's proposal, the most cursory introduction to histology allows one to refute the hypothesis. Cell sizes vary considerably between cell types having identical DNA content in individual metazoan organisms (see Altman and Katz, 1976).

Why does cell size vary? The reasons are likely numerous and varied; however, to date, only theoretical reasons can be given. For example, Szarski (1983) showed mathematically that increases in cell size will reduce the energy cost for maintenance of gradients across membranes. Moreover, one may reason that, for metabolically active tissues such as liver, large cell size should maximize the per-cell metabolic capacity of the tissue. In contrast, a recent study showed an

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inverse correlation between cell size and the structural complexity of the visual centers in amphibian brains (Roth et al., 1994), thereby suggesting that small cell size may favor intricate tissue architecture. Finally, small cell size will increase the number of nuclei per unit volume, and should thereby allow immune tissues such as spleen and thymus to store the maximal number of alternatively recombined genomes.

Several processes which contribute to cell size determination have received previous attention. Some cell types exhibit mechanisms of regulated polyploidization which result in increased cell size (for review see Brodsky and Uryvaeva, 1985). For example, chromosome endoreplication followed by nuclear fusion during liver maturation in rodents leads to polyploidy and may contribute to the large dimensions of adult parenchymal cells (Nadal and Zajdela, 1966). Some processes that might contribute to cell size differences without changing cellular DNA content have also been described. These include the accumulation of large quantities of cell type–specific products such as myosin in muscle (Heywood et al., 1967), and the formation of large vacuoles in certain plant cell types (Matile, 1978). Curiously, however, previous cell size–related investigations have neglected one fascinating observation: different postreplicative somatic cell types in the body produce vastly different amounts of ubiquitous cytoplasmic materials, such as ribosomes, per unit of DNA (see below).

As a result of cell size differences, the relative volumes of nuclear to cytoplasmic compartments exhibit cell type–specific differences. These volume differences are generally accompanied by large differences in RNA:DNA ratios. For example, parenchymal hepatocytes accumulate over ten times more ribosomes per genome-equivalent than splenic lymphocytes (see below) suggesting that expression of the ribosomal genes is cell size dependent. The consequences of such a cell size–dependent regulatory mechanism are expected to be particularly intriguing for genes encoding nuclear transcription factors. Thus, if expression of a gene encoding a transcription factor were affected by cell size in a similar way as the ribosomal genes, the cognate protein would accumulate to higher nuclear concentrations in large cells as compared to small cells. As a consequence, this transcription factor may surpass the threshold concentration for efficient target gene activation only in large cells. Conversely, the expression of a transcription factor which is required at similar concentrations in the nuclei of all cells would have to be insensitive to cell size.

In this study we show that nuclei from different cell types have different intrinsic rates of overall transcription which correlate with differences in RNA accumulation and cell size. These results suggest that metazoans possess a heretofore unconsidered form of overall gene regulation which we entitle "cell size regulation." As a result of cell size differences, higher metazoans, perhaps unlike single-celled eukaryotes, must have independent mechanisms of controlling overall accumulation of ubiquitous cytoplasmic and ubiquitous nuclear macromolecules. The consequences of cell size regulation on expression patterns of nuclear transcription factors are investigated. The results suggest that cell size regulation can largely account for the tissue-specific distribution of the PAR/leucine zipper protein DBP. Conversely, the transcription factors Oct1 and NF-Y accumulate to similar levels in the nuclei of most cell types and thus appear to escape cell size regulation.

Materials and Methods

Animals, Tissue and Explant Preparation, Morphometry, and Kinetic Labeling

Male white rats and MORO mice were used for all studies. Hepatocyte explants were prepared by modifications of the methods of Seglen (1976). For morphometry, livers were perfused via the *vena porta* with 300–400 ml PBS supplemented with 1.1 mg/ml glucose; 3.7 mg/ml sodium bicarbonate; 25 mM Hepes, pH 7.6; 0.5 mM EGTA; preequilibrated with 95% O_2/5% CO_2 (perfusate 1) at a rate of about 25–40 ml/min at 37°C. After starting perfusion, the *vena cava* was cut in the lower back region and the liver was carefully dissected out of the animal and into a small tray maintained at 37°C while perfusion continued. Perfusate 1 was followed by 50 ml of perfusate 2 (1.0 mg/ml collagenase in 150 mM NaCl; 1.1 mg/ml glucose; 5 mM $CaCl_2$; 25 mM Hepes, pH 7.6; 3.7 mg/ml sodium bicarbonate; 0.1 μg/ml DNase 1 [Sigma Chem. Co., St. Louis, MO] preequilibrated with 95% O_2/5% CO_2) at a rate of ~15 ml/min at 37°C. Perfusion was then stopped, the liver was transferred into a covered petri dish, and the dish was floated on a 37°C water bath for an additional 5–10 min. The liver was delicately teased open with needles and cells were washed out with several milliliters of TBS (10 mM Tris, pH 7.5 [at 25°C]; 150 mM NaCl; 0.1 μg/ml DNase 1) at 37°C. Samples containing greater than 20% inviable cells by trypan blue staining were discarded. The cell suspension was passed through a nylon mesh and mixed with two volumes of ice cold 4% glutaraldehyde in PBS. After incubation at 4°C overnight, the solution was decanted, cells were washed twice with PBS and incubated for 20 min in PBS containing 0.5 μg/ml HOECHST's 33258 dye (Sigma Chem. Co.). Cells were washed two more times with PBS and mixed with roughly an equal volume of 50% glycerol; 50 mM Tris, pH 7.5; 0.15 M *n*-propyl gallate (Sigma Chem. Co.); 150 mM NaCl, placed on slides and covered with a free-floating coverslip (cells were not pressed between the slide and cover), and photographed under phase contrast or fluorescence, 40× objective.

During perfusion of the liver with perfusate 1, spleen and thymus were carefully removed and placed into petri dishes. We found that the resilient tegument surrounding these organs will seal tightly around a hypodermic needle. Thus, 0.5 ml of perfusate 2 could be injected into each lobe of the thymus, or 1 ml of perfusate 2 into the spleen. Dishes were covered and floated on a 37°C water bath while liver perfusion was completed. The organs were ruptured with needles and the cells passed through nylon gauze. Trypan blue reliably showed >90% cell viability for both organs. Subsequent steps were identical as for the explanted hepatocytes.

For morphometry, images were photographically enlarged an additional 20×, and cells and nuclei were measured in the largest and smallest dimensions. Gravity dictates that the diameter of the cell in the third dimension will likely be less than or equal to the shortest diameter measured. Thus, volumes were estimated using the equation $v = 4\pi r_l \cdot r_s^2 \cdot 3^{-1}$, where r_l is 1/2 the longest diameter and r_s is 1/2 the shortest diameter.

Cells for kinetic labeling were explanted by a modification of the procedure above. PBS was replaced with 150 mM NaCl, glucose was omitted from perfusates, and perfusate 2 was supplemented with 1 mM hypoxanthine (Sigma Chem. Co.). Labeling mix (2 ml/tissue type: DMEM (Sigma D1152 + 3.7 mg/ml sodium bicarbonate; preequilibrated with 95% O_2/5% CO_2); 10% newborn calf serum (GIBCO BRL, Gaithersburg, MD); 5 μCi/ml [5,6-^{3}H]uridine (New England Nuclear (Boston, MA) NET367, 37.5 Ci/mmol); 1 mM hypoxanthine; 1 mM EGTA; 1 mM $MgCl_2$) was preincubated at 37°C, and 500 μl of the explanted cell suspension was added. At the indicated times, a 250-μl aliquot was transferred into a tube containing 250 μl 1× TES (10 mM Tris, 7.5; 5 mM EDTA; 1% SDS). Samples received 40 μl 3 M sodium acetate, pH 7.0; 1 ml ethanol, and were mixed at 4°C for 1 h followed by 1 h at −70°C. Precipitates were collected by centrifugation and resuspended in 400 μl GT (5 M guanidine thiocyanate; 5 mM sodium citrate, pH 7.0; 0.1 M 2-mercaptoethanol; 0.5% sodium sarkosyl). For incorporation rates, a 200-μl aliquot was precipitated with 10% TCA, washed, and assayed by liquid scintillation. For DNA assays, the remaining 200 μl of sample in GT was diluted to 400 μl with GT, 40 μl 3 M sodium acetate, pH 7.0, was added, samples were extracted twice with Φ/C/I (phenol/$CHCl_3$/isoamyl alcohol; 25:24:1), once with $CHCl_3$, and were precipitated with ethanol. DNA content was determined by the fluorescent assay described below.

For labeling whole organs, the following method was developed. All procedures were performed in a 37°C ambient environment to maintain whole organ temperatures as near 37°C as possible. Liver was perfused first with 6 ml of perfusate 3 (25 mM Hepes, pH 7.6; 150 mM NaCl; 0.5 mM $MgCl_2$; 0.25 mM $CaCl_2$; 50 nM hypoxanthine; 3.7 mg/ml sodium bicarbonate; preequilibrated with 95% O_2/5% CO_2) at roughly 10 ml/min, which was sufficient to visibly eliminate erythrocytes from the organ. The liver was then dissected out of the animal, placed in a petri dish and perfused with 2-ml labeling mix (as above, but supplemented with 0.1% trypan blue; for amino acid incorporation, DMEM + 10% newborn calf serum was replaced by Met-free DMEM + 0.5% FCS and a mixture of ^{35}S-labeled amino acids [77% Met, 18% Cys; New England Nuclear, NEG-072, 1,000 Ci/mmol] replaced the uridine) pumped at 10 ml/min. Labeling mix was then continuously pumped at ~100 μl/min. Trypan blue was chosen as the colorimetric indicator of perfusion homogeneity because it is not assimilated by live cells (Phillips, 1973), and thus is not expected to interfere with metabolism. As medium contained excess nonlabeled Cys, results represent Met incorporation.

For spleen, a 23-gauge needle was slowly inserted through the organ in a petri dish as above while pumping perfusate 3. When near the distal end, the tip of the spleen was clipped and the needle was withdrawn to near the insertion point while pumping labeling mix, which traversed the entire organ en route to the exit at the far tip. Perfusion of labeling mix was continued at 100 μl/min throughout the labeling period.

The efficacy of the method was tested by measuring the amount of total tissue-associated radioactivity per unit DNA in five replicate snippets from perfused organs after quickly rinsing and blotting the surface of each sample. This procedure was expected to remove radioactive fluid from the surface of the tissue, but not remove radiolabel from the interstitial fluids. Liver and spleen showed less than 1.5-fold maximal variation in perfusion homogeneity by this method (average ± SD: liver, 708 ± 124 cpm/ng DNA; spleen, 124 ± 23 cpm/ng DNA). Thymus showed as much as sixfold variation in perfusion homogeneity (175 ± 124 cpm/ng DNA) and therefore was not employed in this study.

At the indicated time points, a small (~100 μl) snippet of a homogeneously blue region of each tissue was excised, rinsed in PBS, blotted briefly on a clean paper wipe, and sonicated in 500 μl TES. Lysates (400 μl) were passed over a 1-ml G25-fine column in TES to remove most nonincorporated radioactivity; the remaining 100 μl of each lysate was extracted and precipitated for fluorescent DNA analysis (see below). The high molecular weight eluate from the column (1 ml) was precipitated with 400 μl 10% TCA; 130 μl 12 M HCl, on ice >30 min, washed with 1 M HCl, and assayed by liquid scintillation.

Preparation of Nuclei and Nuclear Run-on Assays. Nuclei were prepared as described by Tian and Schibler (1991). Nuclear run-on assays were performed as described by Schibler et al. (1983) except that the specific activity of UTP was reduced (50 μM UTP containing 2.5 μCi [^{32}P]/reaction with 30–150 μg DNA-equivalents of nuclei). Because Mn^{++} but not Mg^{++} is present in the run-on reaction, only RNA polymerase II (pol II)[1] elongates nascent transcripts efficiently (Roeder, 1976; Schibler et al., 1983). Run-on reactions (100 μl) were stopped after 13 min at 26°C by adding 520 μl TES, 10 μl 0.5 M EDTA, 50 μl 3 M sodium acetate, pH 7.0, 900 μl ethanol, and mixing at 4°C for 1 h followed by 1 h at −70°C. Control reactions contained 10 μl 0.5 M EDTA and were stopped immediately after adding nuclei. Precipitates were collected and resuspended by sonication in 420 μl TES. A portion (20 μl) was removed, diluted to 400 μl with TES, extracted, and precipitated as above for fluorescent DNA analysis (see below). The remainder (400 μl) was passed over a 1-ml G25-fine/TES column, acid precipitated, washed, and assayed for Cerenkov counts.

Preparation of Total Nucleic Acid and RNA:DNA Ratio Measurements. Numerous technical difficulties needed to be overcome in finding a satisfactory method for measuring RNA:DNA ratios in all tissue types. Indeed, published values for RNA and DNA quantities in tissues vary considerably (Ceriotti, 1955; data compiled by Altman and Katz, 1976; and references therein). The problems fall into four major categories: loss of specific nucleic acid species, interference by nonnucleic acid contaminants (most notably protein or polysaccharides), difficulties in discriminating RNA from DNA, and interference by reagents. None of the published methods that we tested were satisfactory. Therefore, the following method was developed.

Organs were homogenized in greater than 10 vol of either GT or TES at room temperature. NaCl was added to 0.2 M and Tris, pH 8.0, was added to 10 mM, and an equal volume of Φ/C/I was added. Phases were mixed and DNA sheared by sonication. To avoid depletion of DNA, during all extractions any interphase material was harvested with the aqueous phase. After a second extraction the samples were precipitated with ethanol. Pellets were resuspended by brief sonication in TCS (10 mM Tris, pH 7.5; 0.5 mM $CaCl_2$; 1% SDS), proteinase K was added to 40 μg/ml, and samples were incubated at 50°C for 30 min. NaCl was added to 0.2 M, EDTA to 1 mM, samples were extracted twice with Φ/C/I, once with $CHCl_3$, and precipitated with ethanol. At this point, trace nucleosides and mononucleotides have been depleted by extractions and precipitations, lipids and soluble proteins have been extracted, and structural proteins have been digested and subsequently extracted. Samples are expected to contain all of the organ's polymeric nucleic acid, as well as some complex polysaccharides, such as glycogen.

Precipitates were collected by centrifugation and resuspended by brief sonication in water. The amount of glycogen in liver samples at this point is too high to allow absorbance measurements, and can vary more than 10-fold between liver preparations depending on factors such as the time of harvest. Therefore, after sonication at room temperature, the aqueous samples were clarified in a microcentrifuge at room temperature for 3 min. The large glycogen pellet in liver samples exhibited no coprecipitated nucleic acid by gel electrophoresis (not shown). The clarity of the supernatant suggested that roughly 80% of the glycogen was removed by this procedure.

The following method for measuring RNA in the samples was developed. An aliquot of the clarified aqueous solution was adjusted to 500 μl with water. A standard curve containing known amount of pure RNA and control samples containing known amounts of pure DNA were prepared in parallel. Samples received 500 μl 1 M NaOH and were incubated at 50°C for 1 h to completely hydrolyze RNA without hydrolyzing DNA. After samples were cooled 3 min on ice, 130 μl of ice-cold 12 M HCl was added, and samples were incubated on ice 30 min to precipitate glycogen and DNA (TCA absorbs UV light and would interfere with subsequent measurements). The short incubation time and low temperature prevented depurination of DNA (confirmed by DNA controls). Samples were clarified by centrifugation; the supernatant was transferred to a fresh tube, heated for several minutes at 75°C (to acid hydrolyze residual polysaccharides), and absorbance at 260 nm was measured. Controls indicated less than 0.1% interference by DNA. Treatment of these lysates with orcinol (Sigma Chem. Co.; Ceriotti, 1955) indicated that although some glycogen-derived sugars persist in the supernatants, they no longer interfered with ultraviolet absorbance measurements (not shown).

To measure DNA in the aqueous samples, a fluorescent assay was used. The assay is based on DNA-specific intercalation and fluorescence of HOECHST's 33258 dye by native DNA. It is important that samples never be heat or base denatured. The method we used was as described by Labarca and Paigen (1980), except that we find optimal DNA-specific fluorescence with an excitation wavelength of 352 nm, an emission wavelength of 450 nm, and slit widths of 10 mm each on a LS-5B luminometer (Perkin-Elmer Corp., Norwalk, CT). Controls using either yeast RNA (primarily tRNA) or rat kidney RNA purified through a CsCl cushion (primarily rRNA) show less than 0.1% interference. Purified rat glycogen at a concentration that yields an absorbance reading equivalent to 1 μg/ml DNA shows 2.1% interference (equivalent to 21 ng/ml DNA). In combination with the roughly fivefold depletion of glycogen by the centrifugation step above, the assay is ~200-fold less sensitive to glycogen (and likely other polysaccharides) than direct absorbance assays; this was judged sufficient for our studies.

RNA Purification and RNase Protection Assays. A modification of the method of Glisin et al. (1974) was used to purify RNA for RNase protection assays. Organs were homogenized in >10 vol of GT (5 M guanidine thiocyanate; 5 mM sodium citrate, pH 7.0; 0.1 M 2-mercaptoethanol; 0.5% sodium sarkosyl), adjusted to 0.4 g/ml CsCl, and sonicated to reduce viscosity. Samples (5 ml) were layered onto 7-ml cushions of 5.7 M CsCl; 1 mM EDTA in 12 ml 50Ti tubes, and sedimented for 16 h at 30,000 rpm, at 20°C (Beckman Instrs., Fullerton, CA). Under these conditions, RNA sediments almost quantitatively (gel electrophoresis of samples shows mRNA, rRNA, and tRNA), but glycogen in liver samples forms a tight band ~1 cm from the bottom of the tube.

RNase protection assays were as described previously (Schmidt and Merrill, 1989) except that hybridization temperatures were reduced to 52°C. To confirm data, RNase protection assays, except those using the NF-Ya probe (which hybridizes only to mouse NF-Ya mRNA), were repeated using RNA samples from both mouse and rat. However, the only data shown from cross-species probe hybridization is that for GAPDH in Fig. 8. For cross-species assays, conditions were as normal except that RNase

1. *Abbreviations used in this paper*: EMSA, electrophoretic mobility shift assay; pol II, RNA polymerase II.

digestion temperatures were lowered from 37 to 22°C. The following RNA probes were used in the work presented: NF-Ya, a transcript complementary to mouse mRNA sequences between +1076 and +1294 (Li et al., 1992; note, this probe does not discriminate between the alternatively spliced isoforms of NF-Ya mRNA, so results represent the sum of both isoforms); NF-Yb, a transcript complementary to mouse mRNA sequences between +266 and +491 (Li et al., 1992); Oct1, a transcript complementary to mouse mRNA sequences between +1175 and +1511 (Suzuki et al., 1993); DBP, a transcript complementary to rat mRNA sequences between +370 and +505 (Mueller et al., 1990); GAPDH, a transcript colinear with a 200-base EcoRI–AccI region of the rat cDNA (Fort et al., 1985); albumin, a transcript complementary to rat mRNA sequences between +1320 and +1720. To confirm the specificity of the signals detected, the following probes directed to distinct regions of the mRNAs of interest were hybridized to RNA samples from large- and small-celled tissues sources: NF-Ya, a transcript complementary to rat mRNA sequences between +1 and +144 (Li et al., 1992); NF-Yb, a transcript complementary to mouse mRNA sequences between +492 and +752 (Li et al., 1992); Oct1, a transcript complementary to mouse mRNA sequences between +1 and +233 (Suzuki et al., 1993); DBP, a transcript colinear with a genomic fragment spanning from +600 to +196 (Wuarin and Schibler, 1990). In all cases, the cell size-dependent expression patterns for these mRNAs were corroborated by both probes (not shown).

Polysome Fractionation. Polysome fractions were prepared by a modification of the methods of Schmidt and Merrill (1991). Preliminary analyses indicated that RNA harvested from polysome gradients of kidney, spleen, brain, heart, and to a somewhat lesser extent lung, was degraded as visualized by denaturing gel electrophoresis (not shown). Only liver, which is rich in endogenous RNase inhibitors (Gribnau et al., 1969), and testis yielded intact RNA. Thus, these two tissues were chosen for analysis of the NF-Yb polysome distribution.

Mueller et al. (1990) demonstrated that DBP mRNA is expressed in all tissues except testis. Thus, an alternative system was required to measure the polysomal distribution of DBP mRNA. For these studies, advantage was taken of the abundance of RNase inhibitors in liver and the circadian expressing pattern of DBP mRNA. DBP mRNA is undetectable in RNA samples harvested in the early morning hours (<1% evening levels; Wuarin and Schibler, 1990; and data not shown). Thus, morning liver provided a rich source of DBP mRNA-free RNase inhibitor (the cost of this amount of commercial inhibitor would be prohibitive). Preliminary analysis demonstrated that fresh and frozen polysomal lysates gave indistinguishable polysomal gradients in terms of ultraviolet elution profiles, RNA integrity, and the distribution of specific messages using lysates from either rat liver or tissue culture (Schmidt and Merrill, 1991; and data not shown). Therefore, liver was harvested from a rat killed at 8:00 am, homogenized at 4°C in 1 ml/g tissue of 20 mM Tris, pH 7.6 (at 25°C); 150 mM NaCl; 10 mM $MgCl_2$; 10 mM DTT; 20 μg/ml cycloheximide in a motor-driven Teflon/glass homogenizer. Triton X-100 was added to 1%, nuclei were pelleted in an HB-4 rotor at 0°C, 3 min, 3,000 rpm (Sorvall Instrs., Wilmington, DE), and the supernatant was stored at −70°C until evening. At 5:00 pm, roughly 1 g of liver or lung tissue from a littermate was homogenized in 10 ml each of the morning lysate (lung was chosen because preliminary experiments suggested that RNase levels were somewhat lower in lung lysates as compared to spleen, see above). Nuclei were sedimented as above, and 0.5 ml of each lysate was layered on 15–50% sucrose gradients and sedimented as described (Schmidt and Merrill, 1991). A control gradient containing only the morning liver lysate confirmed that the DBP signal arose from the evening liver or lung samples (not shown).

Nuclear/Cytoplasmic RNA Preparation. Nuclei from liver and spleen of mice and rats killed at 3:00 pm (circadian peak of DBP transcription) were prepared by a modification of the citric acid method (Wuarin and Schibler, 1990). The low pH of the citric acid homogenization buffer allowed purification of intact nuclear RNA by inhibiting cellular RNases. After homogenization and filtration of tissues and sedimentation of nuclei through two 30% sucrose pads as described (Wuarin and Schibler, 1990), nuclear pellets were dispersed, nuclei were rapidly resuspended in 10 ml of ice cold NFB (40% glycerol, 50 mM Tris, pH 8.3 (at 25°C), 5 mM $MgCl_2$, 0.5 mM EDTA) and pelleted for 3 min, 0°C, 3,000 rpm in a Sorvall HB-4 rotor. Supernatants were removed and nuclear pellets were immediately sonicated in 6.5 ml GT. CsCl (3.2 g) was added and volumes were adjusted to 8 ml. Lysates were layered on 4-ml cushions of 5.7 M CsCl; 1 mM EDTA, and RNA was pelleted in a Beckman Ti50 rotor at 39,000 rpm for 13 h, 20°C. RNA integrity was confirmed on stained formaldehyde/agarose gels.

Other Methods and Reagents. Nuclear extracts were prepared and electrophoretic mobility shift assays (EMSA) performed by the methods of Lavery and Schibler (1993) using the rat albumin promoter C site oligonucleotide described by Wuarin et al. (1990) for NF-Y and the octamer-containing oligonucleotide described by Müller et al. (1988) for Oct1. Western blots were as per Descombes et al. (1990), except for the analysis of pol II large subunit, nuclear DNA was sheared at 37°C, 15 min, with 0.5 U/μg DNA of RQ1 DNase (Promega Corp., Madison, WI) in 1 mM $MgCl_2$ before adding SDS/dye mix and loading on gel, or as specified in figure legends.

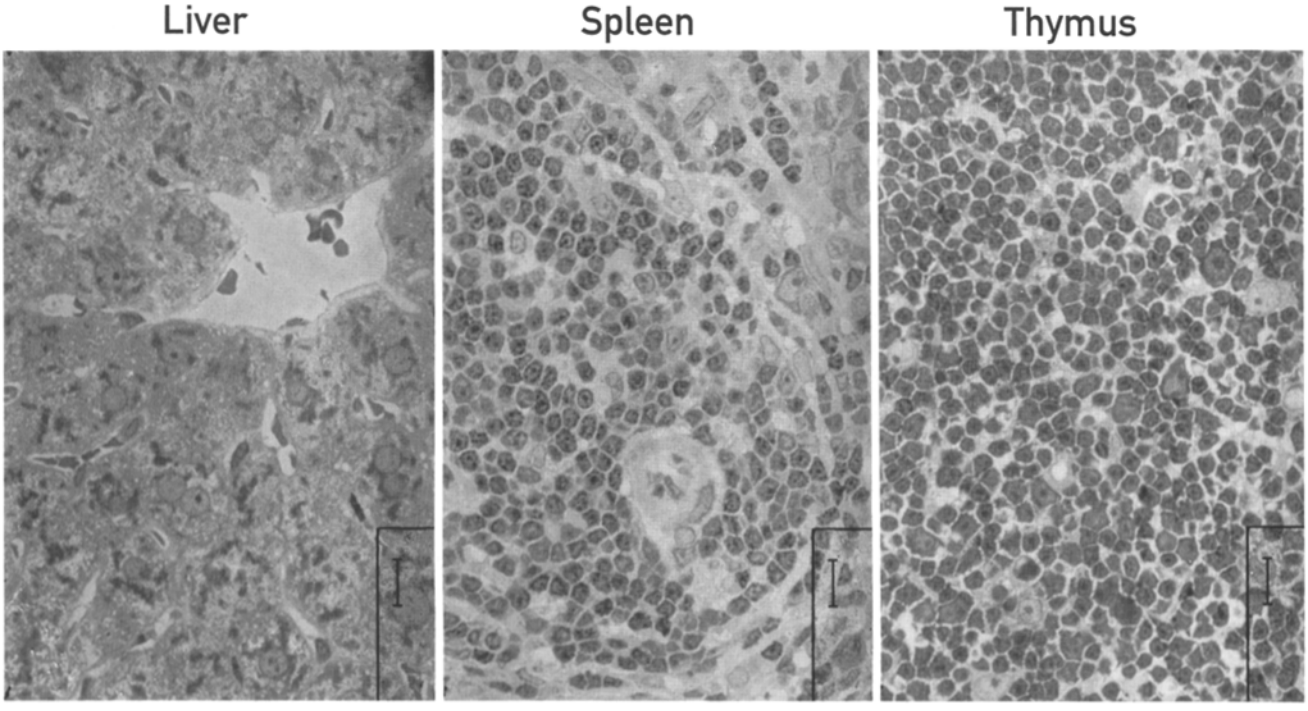


Figure 1. Liver, spleen, and thymus histology in 5-wk-old rats. Semithin (0.5 μm) sections of fixed tissues embedded in plastic were prepared, stained with toluidine blue, and photographed. All frames are the same magnification.

Results

Cell Size Regulation

Cell size varies greatly among cell types in metazoans (cell sizes from various species and tissue types are compiled in Altman and Katz [1976]). This is exemplified by a microscopic comparison between tissue sections from liver, spleen, and thymus (Fig. 1). These three organs were chosen because, as seen in the micrographs, each is populated predominantly by cells of similar size, but average cell sizes differ vastly between the organs. In comparing cells in these three organs, several aspects deserve attention: first, the amount of nuclear material per cell can vary (for review see Brodsky and Uryvaeva, 1985). Thus, due to polyploidy, most liver nuclei are severalfold larger than the diploid nuclei in spleen and thymus and several binucleate cells are observed in the liver micrograph. Second, the nuclei of liver cells exhibit less heterochromatin than spleen or thymus nuclei. The high proportion of decondensed euchromatin suggests that total transcriptional activity might be greater in liver (for review see Bouteille et al., 1974). Finally and most importantly for this study, the micrographs show that the greatest difference in cell sizes resulted from a greater average cytoplasmic:nuclear volume ratio for liver than for spleen or thymus. Consistent with this, Table I shows that, in comparing six different organs, the wet mass of tissue per DNA equivalent varies over a 20-fold range. We hypothesized that the difference in cytoplasmic:nuclear ratios between cell types may represent a regulated difference in the ability of nuclei to direct the steady-state accumulation of ubiquitous cytoplasmic material.

RNA:DNA ratios were measured in eight organs to biochemically quantitate relative accumulation of ubiquitous cytoplasmic material between different tissue types (Fig. 2). The amount of total RNA per genome-equivalent varied over a 16-fold range, with liver having an RNA:DNA ratio of 4.7 and thymus a ratio of 0.3. Because the bulk of total RNA is rRNA, these data indicate that cells in the liver, on average, accumulated roughly 16-fold more ribosomes per unit DNA than cells in the thymus.

For several reasons, we consider the RNA:DNA ratio the most reliable measure of differences in per-genome accumulation of ubiquitous cytoplasmic products. First, it is the most direct measure of the ability of the genome to direct the accumulation of RNA. Thus, although a polyploid cell might produce more RNA as a result of having more gene copies, RNA:DNA ratios reflect relative per-genome RNA accumulation. Second, the RNA:DNA ratio provides a reproducible measure of average cytoplasmic:nuclear content within an organ which can be compared to other biochemical properties of the tissue, such as specific mRNA or protein levels, and transcription or translation rates. Last, although tissue-specific functions may require different amounts of ribosomes, tRNAs, and total mRNA, which will affect RNA:DNA ratios, the RNA:DNA ratios are blind to the accumulation of abundant tissue-specific products such as fat droplets in adipocytes, contractile proteins in myotubes, or extracellular materials in connective tissues or brain. The methods we establish here may provide a reliable means of measuring RNA:DNA ratios in most biological samples.

It was possible that differences in RNA:DNA ratios might represent differences in RNA concentration between cell types and not differences in volume. To determine whether RNA:DNA ratios reflect differences in cell size, measurements were made on explanted hepatocytes, splenocytes, and thymocytes (Fig. 3). Total nucleic acid was prepared from a portion of the cells for biochemical analysis of RNA:DNA ratios in the explants. The remainder of the cells were fixed while in suspension and nuclear and cellular volumes were estimated microscopically. Comparison of the biochemical and volumetric data in Fig. 3 *A* indicates that relative RNA:DNA ratios correlated reasonably well with relative cell sizes between these three cell types.

In conclusion, the results above indicate that different cell types accumulate vastly different steady-state levels of total RNA per genome-equivalent. The differences in RNA:DNA ratios roughly correlated to differences in cytoplasmic:nuclear volume ratios. We entitle the mechanism responsible for this difference, cell size regulation. To accumulate different steady-state levels of cellular RNA, cells must differ in

Table I. Per-genome Equivalent of Wet Tissue Mass for Various Organs

Tissue	Tissue mass/DNA	
	mg/mg	*(% liver)*
Liver	870	(100)
Lung	190	(22)
Kidney	480	(55)
Spleen	42	(5)
Brain	890	(103)
Thymus	32	(4)

The indicated organs were harvested from an adult mouse, blotted briefly on clean paper wipes, and weighed. Total nucleic acid was extracted from each sample and the DNA content was determined.

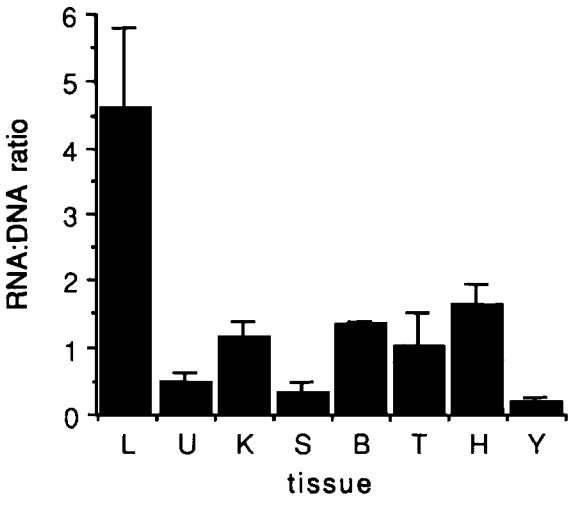
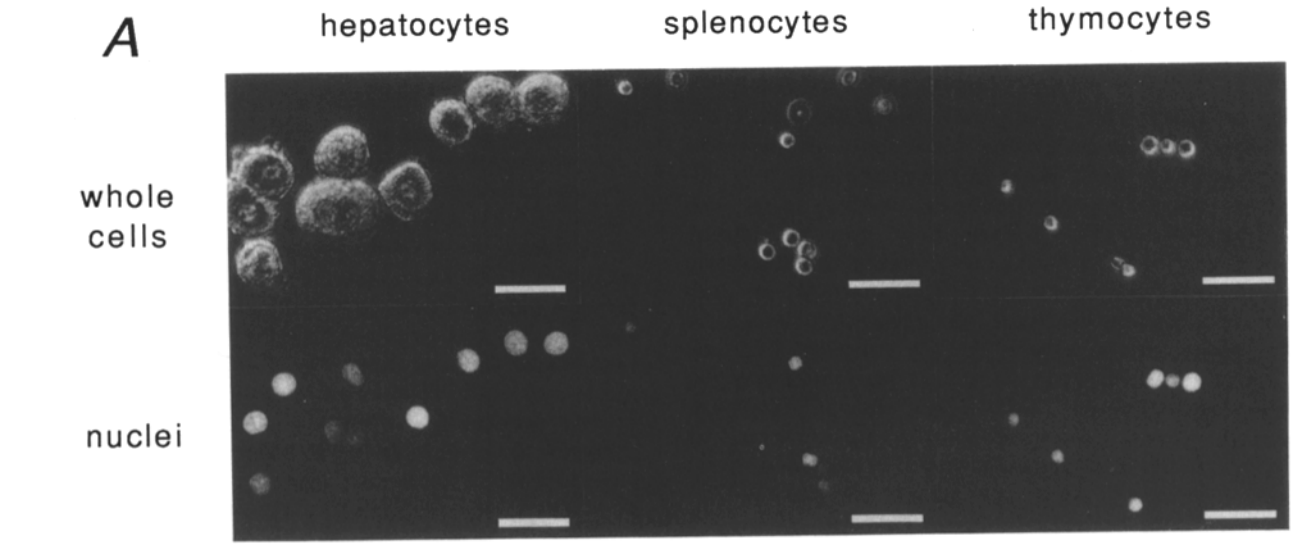


Figure 2. RNA:DNA ratios in adult organs. Total nucleic acid was prepared and used to measure RNA:DNA ratios from three rats (ages 6, 7, and 9 wk) and four mice (ages 5, 7, 12, and 13 wk; all samples were prepared separately). *L*, *U*, *K*, *S*, *B*, and *T* were harvested from all animals; *H* and *Y* were only harvested from rats. Data represent average values; fine bars represent one standard deviation. The abbreviations *L*, liver; *U*, lung; *K*, kidney; *S*, spleen; *B*, brain; *T*, testis; *H*, heart; and *Y*, thymus, are used here and in subsequent figures.



$\frac{\text{cytoplasm}}{\text{nucleus}}$	$\left(\frac{\text{vol}}{\text{vol}}\right)$	13.7	0.8	0.4
$\frac{\text{cell}}{\text{nucleus}}$	$\left(\frac{\text{vol}}{\text{vol}}\right)$	14.7	1.8	1.4
$\frac{\text{RNA}}{\text{DNA}}$	$\left(\frac{\text{wt}}{\text{wt}}\right)$	6.3	0.8	0.3
$\frac{\text{transcription}}{\text{DNA}}$	$\left(\frac{\text{cpm}}{\mu\text{g}\cdot\text{min}}\right)$	980	160	37

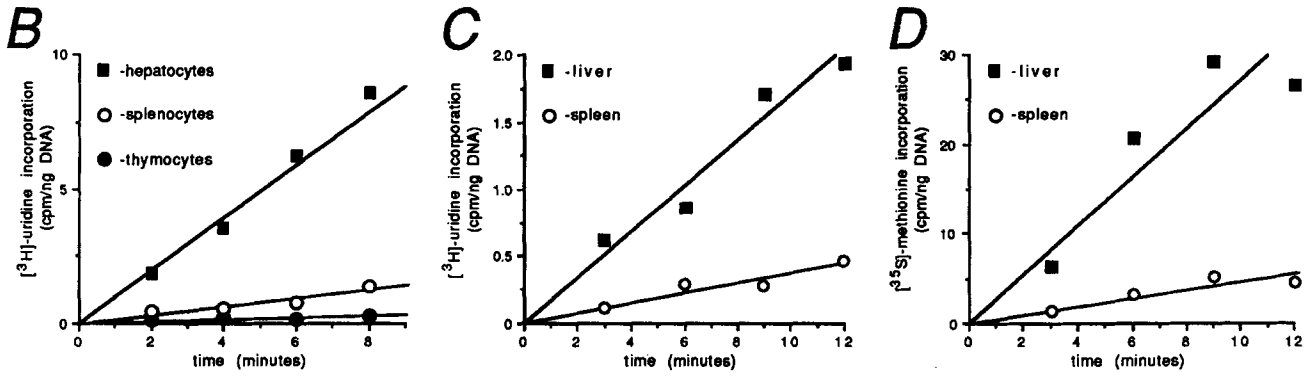


Figure 3. Cell sizes, RNA:DNA ratios, and in vivo labeling kinetics for liver, spleen, and thymus. (*A*) Measurements on explanted cells. Hepatocytes, splenocytes, and thymocytes were explanted from 7-wk-old rat tissues. All photo frames are at the same magnification. Upper frames are phase-contrast micrographs; lower frames are HOECHST's dye fluorographs to allow measurements of nuclear volumes. In each case, upper and lower photos are of the same frames; erythrocytes in the splenocyte and thymocyte preparations exhibit no nuclei. Volumetric data represent average values for 15 hepatocytes, 14 splenocytes, and 32 thymocytes. The RNA:DNA ratios were determined on a separate aliquot of the same explants before fixation. Transcription rates represent the slope of the curves in *B*, and are listed here to facilitate comparisons. (*B*) In vivo uridine incorporation kinetics by explanted cells. Explanted cells were incubated in the presence of labeled uridine to measure overall transcription rates. Time points were taken at 2-min intervals and analyzed for acid-precipitable radioactivity and DNA content. Values represent specific radioactivity per input DNA. (*C*) Uridine incorporation by whole perfused liver and spleen. Snippets of each organ were harvested at 3-min intervals after initiation of perfusion with radiolabeled uridine. Values represent the acid-precipitable radioactivity per unit DNA in each sample. (*D*) Amino acid incorporation by whole perfused liver and spleen. As in *C*, except that labeling mix contained radiolabeled amino acids.

either the rate at which they synthesize RNA, the rate at which they degrade RNA, or both.

Large Cells Synthesize More RNA Than Small Cells. Kinetic studies of precursor incorporation rates were used to distinguish between the different mechanisms that might account for cell size regulation. Three approaches were employed in these studies: in vivo labeling using explanted cells, in vivo labeling using whole perfused organs, and in vitro labeling using isolated nuclei.

In the first approach, uridine incorporation was measured in cells explanted from liver, spleen, and thymus (Fig. 3 *B*). Uridine incorporation per unit time was divided by the mass of DNA in each sample as an indication of the per-genome transcription rates. The results show that the transcription rate per DNA in hepatocytes was ~6- and 26-fold greater than rates in splenocytes and thymocytes, respectively (tabulated in Fig. 3 *A*). Significantly, if uridine incorporation rates per unit DNA are divided by the RNA:DNA ratios, all values fall within a twofold range (hepatocytes: 156 cpm/μg RNA·min; splenocytes: 200 cpm/μg RNA·min; thymocytes: 310 cpm/μg RNA·min). Thus, differential transcription rates, rather than differential RNA stability, largely accounted for the different RNA:DNA ratios.

As a second approach to measuring precursor incorporation kinetics, whole liver and spleen were continuously perfused with labeling media and small tissue pieces were harvested at various time points. The amount of incorporated radioactivity was normalized to the amount of DNA in each sample. The results show that the uridine incorporation rate per unit DNA in liver was 5.7-fold greater than that in spleen (Fig. 3 *C*). The results confirm that RNA synthesis can account for most of the difference in RNA:DNA ratios between liver and spleen.

Methionine incorporation rates were measured in the perfused organs as an indication of translational efficiencies. The results indicate that liver incorporated methionine at a per-genome rate that was 6.2-fold greater than that in spleen (Fig. 3 *D*). Comparison of methionine and uridine incorporation rates between the perfused organs shows that rates of protein and RNA synthesis correlated well (compare Fig. 3, *C* and *D*). Division of the per-DNA methionine incorporation rates by the RNA:DNA ratios indicates that per-RNA rates of protein synthesis varied by less than twofold between liver and spleen.

As a third approach to determining the level of regulation of RNA:DNA ratios in different tissue types, in vitro incorporation of UTP was measured during run-on transcription in isolated nuclei under conditions in which only RNA polymerase II (pol II) efficiently elongates nascent transcripts (Table II; Gariglio et al., 1981). Relative differences in run-on transcription rate per unit DNA roughly reflected differences in RNA:DNA ratios between the six tissue types examined; only small differences in total RNA accumulation per transcription activity could be measured (last column in Table II). In conclusion, all three approaches suggest that regulation of overall transcription rates, rather than of overall RNA stabilities, largely account for the differences we measured in RNA:DNA ratios.

Accumulation of RNA Polymerase II in Organs with Differences in Cell Size. Although differences in pol II activity alone cannot account for differences in cellular RNA:DNA ratios, the data in Table II indicate the pol II activity is cell size–dependent. Therefore, we asked whether these activity differences could be accounted for simply by differences in levels of pol II protein. Western blots for pol II large subunit show that nuclear levels of the protein do not correlate well to relative RNA:DNA ratios (Fig. 4). Thus, although nuclei of small cells, such as spleen and lung, exhibited less pol II than those of larger cells, such as liver, kidney, and brain, liver pol II levels did not appear higher than those in kidney. Also, testis (RNA:DNA = 1.1) exhibited at least as much pol II per unit DNA as liver (RNA:DNA =

Table II. In Vitro RNA Synthesis in Isolated Nuclei

Tissue	RNA/DNA	Relative pol II transcription	Relative pol II transcription / RNA/DNA
	mg/mg (% liver)		
Liver	4.71 (100)	100	1.0
Lung	0.56 (12)	18	1.5
Kidney	1.24 (26)	41	1.6
Spleen	0.42 (9)	10	1.1
Brain	1.44 (31)	34	1.1
Thymus	0.30 (6)	9	1.5

Run-on transcription rates were determined in isolated nuclei under conditions which favor pol II-specific elongation. Values are presented as percent of the liver value to facilitate comparison to RNA/DNA values. Control reactions were stopped immediately and thus represent a zero time point. The following specific values are given to show representative signal to background: liver: experimental, 52.3 μg DNA-equivalents of nuclei yielded 51,000 cpm (Cerenkov); control, 58.1 μg DNA-equivalents of nuclei yielded 662 cpm. Spleen: experimental, 57.6 μg DNA-equivalents of nuclei yielded 6,240 cpm; control, 57.3 μg DNA-equivalents of nuclei yielded 357 cpm. The correlation between RNA/DNA ratios and overall pol II transcription rates is exhibited in the last column by division of the transcription rate values by the RNA/DNA ratio determinations.

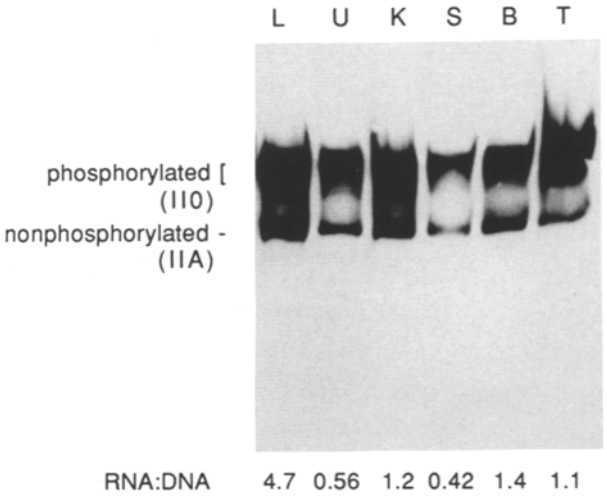


Figure 4. RNA polymerase II large subunit in nuclei from various tissue sources. A Western blot of a 5.2% polyacrylamide gel containing 20 μg DNA-equivalents per lane of whole DNase-treated nuclei from the indicated rat tissues was visualized using mouse monoclonal antibody to the CTD polypeptide of the large subunit of RNA polymerase and the "ECL" method (Amersham Corp., Arlington Heights, IL). The top of the photo corresponds to the top of the running gel; bands corresponding to phosphorylated (IIO) and underphosphorylated (IIA) forms are indicated. The RNA:DNA ratios for the respective tissues are listed below the autoradiogram to ease comparison to signal intensities.

4.7). It may be interesting that nuclear levels of underphosphorylated pol II molecules (IIA) more closely matched differences in pol II-dependent transcription rates (Table II). Pol IIA has been proposed to play the primary role in initiating transcription (for review see Corden, 1993). However, until we can exclude the possibility that the equilibrium between pol IIA and pol IIO was altered during the isolation of nuclei, we cannot be certain that the observed correlation is significant. In conclusion, overall levels of pol II probably do not account entirely for cell size–dependent transcription rates.

Gene Expression As a Consequence of Cell Size

Genes Encoding Ubiquitous Transcription Factors Escape Cell Size Regulation. Because cell sizes differ, two general types of ubiquitous macromolecules can be defined. Ubiquitous nuclear molecules, exemplified by DNA and histones, are generally equally represented in most cells. In contrast, cellular levels of ubiquitous cytoplasmic macromolecules vary with cell size. This simple observation necessitates that genes encoding ubiquitous nuclear and ubiquitous cytoplasmic products be subject to distinct mechanisms of regulation. Because large and small cells accumulate similar steady-state numbers of most ubiquitous nuclear macromolecules per DNA, cell size regulation, the mechanism that directs greater synthesis of ubiquitous cytoplasmic macromolecules in cells with high RNA:DNA ratios, must be either ignored or overridden by genes encoding ubiquitous nuclear products. To test this prediction, we investigated expression patterns of three genes encoding ubiquitous nuclear proteins.

As most nuclear macromolecules are synthesized in nondividing cells only to compensate for turn over, and because synthesis rates equal degradation rates at steady-state, short-lived ubiquitous nuclear macromolecules were sought for this study. We reasoned that the ubiquitous transcription factors, unlike histones and perhaps most structural nuclear proteins, would have sufficiently short halflives to allow measurement of compensatory synthesis rates in postrepli-

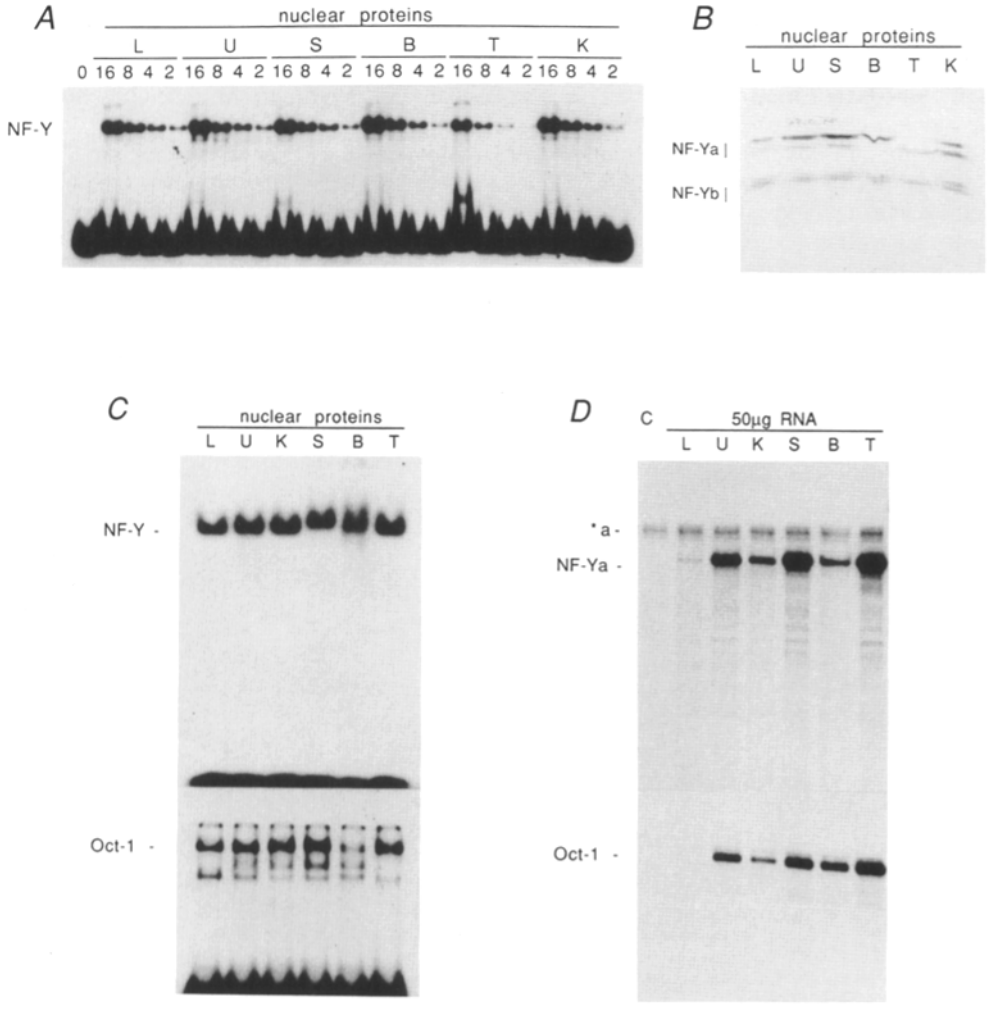


Figure 5. NF-Y and Oct1 in tissues. (*A*) NF-Y-specific DNA-binding activity in nuclei. The indicated volumes of nuclear extracts (all corresponding equal DNA-equivalents of nuclei/vol) were used in an EMSA with an oligonucleotide probe corresponding to the rat albumin promoter C site. (*B*) NF-Ya and NF-Yb protein levels. A Western blot (13% polyacrylamide gel) containing 50 μg DNA-equivalents/lane of nuclear proteins from the indicated tissues was visualized using a mixture of rabbit polyclonal antibodies raised against NF-Ya and against NF-Yb followed by goat-anti-rabbit alkaline phosphatase–conjugated second antibody. (*C*) Comparison of relative levels of NF-Y- and Oct1-specific DNA-binding activity in tissues. Equal DNA-equivalents of nuclear extracts from the indicated tissues were used in EMSA assays with the albumin promoter C site oligonucleotide (upper autoradiogram) or octamer-containing oligonucleotide (lower autoradiogram) in parallel to allow direct comparison of relative levels of the two DNA-binding activities in nuclei. (*D*) mRNA levels for NF-Ya and Oct1 in tissues. RNase protection assays were performed using 50 μg per assay of total RNA harvested from the indicated mouse tissues. For both mRNA species, 5 fmol of the respective probe (55 Ci/mmol) was included in each hybridization. The NF-Ya probe hybridizes to both isoforms of NF-Ya mRNA (see text and Materials and Methods), such that the signal observed represents the sum of both mRNA isoforms in each sample. Abbreviations: **a*, residual nondigested NF-Ya riboprobe; *C*, control lanes containing 50 μg of yeast RNA to show signal specificity.

cative cells. The genes encoding Oct1, NF-Ya, and NF-Yb were chosen for this study. Oct1 is a homodimeric transcription factor which is expressed at similar levels in nuclei from most tissue and tissue-culture sources (Barberis et al., 1987; Miwa et al., 1987; Schöler et al., 1989; Sturm et al., 1988; Suzuki et al., 1993; Tanaka et al., 1992). The CCAAT-binding transcription factor NF-Y (CP1, CBF, HAP2/3/4) is a heteromer containing subunits NF-Ya, NF-Yb, and at least one other subunit (Chodosh et al., 1988*a*,*b*; Forsburg and Guarente, 1989; Hatamochi et al., 1988; Hooft van Huijsduijnen et al., 1990; Maity et al., 1992). Two isoforms of NF-Ya and two isoforms of NF-Yb exist in nuclei from different tissue and cell types (Hooft van Huijsduijnen et al., 1990; Li et al., 1992; Maity et al., 1992; Vuorio et al., 1990). The two isoforms of NF-Ya arise from differential splicing (Li et al., 1992) whereas the isoforms of NF-Yb result from posttranslational modification (Maity et al., 1992). By all assays to date, both isoforms of NF-Ya and both isoforms of NF-Yb exhibit indistinguishable activities (Li et al., 1992; Maity et al., 1992; Mantovani et al., 1992), and therefore were considered equivalent here.

We first quantitated the levels of NF-Y and Oct1 in nuclei from different tissue sources to confirm that nuclei contain similar numbers of these ubiquitous transcription factors. Electrophoretic mobility shift assays (EMSA) using serial dilutions of nuclear extracts demonstrated that NF-Y–dependent DNA-binding activity levels were similar in the different tissue types (Fig. 5 *A*). However, because NF-Y is a heteromeric factor, it was possible that the levels of any one subunit could limit NF-Y activity in different nuclei sources. Therefore, levels of NF-Ya and NF-Yb protein were measured by Western blot analysis (Fig. 5 *B*). NF-Ya and NF-Yb protein levels were both similar in nuclei from all six tissue types (within roughly twofold for the sum of the isoforms for either protein). Because Oct1 binds DNA as a homodimer, it is possible to estimate Oct1 protein levels directly from EMSA analysis (Barberis et al., 1987; Miwa et al., 1987; Ossipow et al., 1993; Schöler et al., 1989; Sturm et al., 1988; Suzuki et al., 1993; Tanaka et al., 1992). Comparison of Oct1-specific to NF-Y-specific DNA-binding activity demonstrated that, with the exception of the previously described brain-specific deficiency of Oct1 activity (Schöler et al., 1989), nuclei from different tissues contained similar levels of Oct1- and NF-Y–specific DNA-binding activities (Fig. 5 *C*). As Oct1, NF-Ya, and NF-Yb were present at similar numbers per DNA-equivalent in tissue types with vastly different RNA:DNA ratios, our results confirm that these proteins are not subject to cell size regulation.

Regulation of Nuclear NF-Ya, NF-Yb, and Oct1 Protein Levels. Levels of mRNAs encoding NF-Ya, NF-Yb, and Oct1 were quantitated next to determine whether they also were cell size–independent. To correctly interpret mRNA analyses between different tissue types, the RNA:DNA ratios of the tissues were taken into consideration. As a result of the differences in RNA:DNA ratios, a given mass of total RNA will represent 11-fold more genome-equivalents of spleen tissue than of liver. The comparison of 50 μg of total RNA from different tissue types in Fig. 5 *D* shows the relative concentrations of NF-Ya and Oct1 mRNA. The signal intensities must be multiplied by the RNA:DNA ratios to find the per-genome number of mRNA molecules. Because the relative signal intensities for both NF-Ya and Oct1 mRNA were

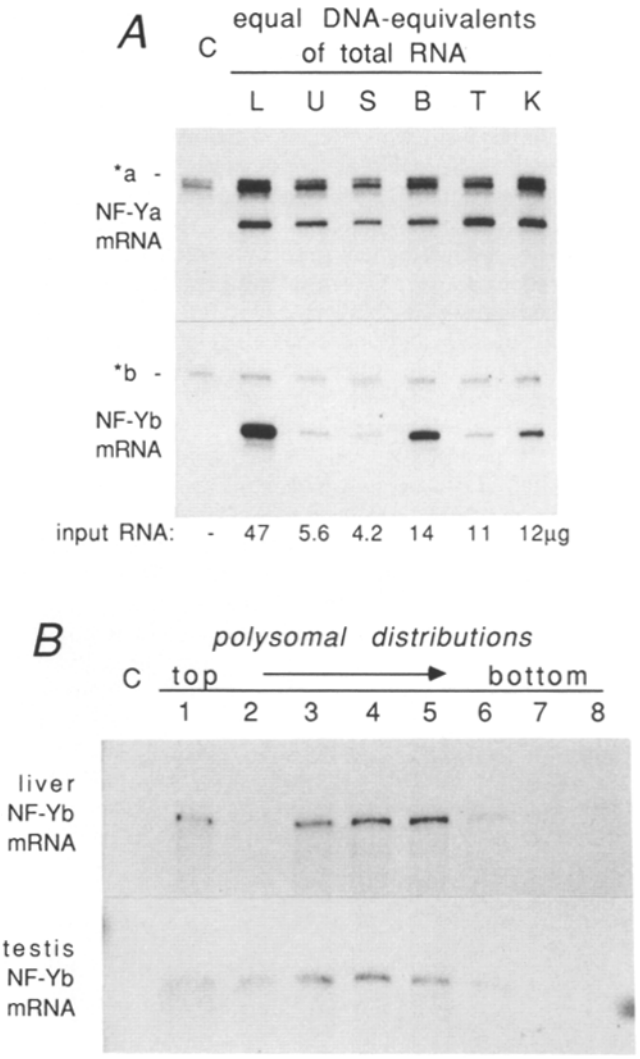


Figure 6. NF-Ya and NF-Yb regulation in tissues. (*A*) NF-Ya and NF-Yb mRNA levels per-genome-equivalent in mouse tissues. RNA:DNA ratios (Fig. 2) were used to determine equal DNA-equivalents of RNA (listed below autoradiograms) and this amount of RNA was supplemented with yeast RNA to 100 μg. RNase protection assays were as above. (*B*) Polysomal distribution of NF-Yb mRNA in liver and testis. Cytoplasmic preparations from liver and testis were sedimented in sucrose gradients. An aliquot (25%) of RNA purified from each fraction was supplemented with 50 μg of yeast RNA and hybridized to 5 fm of the NF-Yb riboprobe. The control lanes contained probe hybridized to 100 μg yeast RNA. RNase protection conditions were as above. Abbreviation: **b*, residual nondigested NF-Yb probe.

roughly inversely proportional to the RNA:DNA ratios for these tissues (compare Figs. 2 and 5 *D*), one may predict that all six tissues contain an equal number of NF-Ya and Oct1 mRNA molecules per genome-equivalent. This prediction is tested for NF-Ya in Fig. 6 *A*. The amount of total RNA that corresponds to 10 μg DNA-equivalents of tissue was included in each hybridization. The results confirm that all tissues contained a similar number of NF-Ya mRNA molecules per genome-equivalent. Thus, levels of NF-Ya mRNA (Fig. 6 *A*, *upper panel*) were roughly proportional to levels of NF-Ya protein (Fig. 5 *B*) in all tissues. In contrast, per-genome levels of NF-Yb mRNA (Fig. 6 *A*, *lower panel*) varied con-

siderably and were not proportional to per-genome levels of NF-Yb protein (Fig. 5 *B*). Rather, NF-Yb mRNA was expressed at roughly the same concentration per total RNA in all cell types (autoradiogram not shown, but note that the relative signal intensities in Fig. 6 *A* are roughly inversely proportional to RNA:DNA ratios). The relative cellular mRNA and protein or DNA-binding activity levels for Oct1, NF-Ya, and NF-Yb are shown in Fig. 7, *A*–C. Each panel compares total RNA content to specific mRNA and protein levels for each organ type. This graphic presentation demonstrates that levels of NF-Ya mRNA, Oct1 mRNA, NF-Ya protein, NF-Yb protein, and Oct1 DNA-binding activity were insensitive to the large differences in total RNA content between the tissues. In contrast, levels of NF-Yb mRNA corresponded fairly well to cellular RNA content (Fig. 7 *C*). Because NF-Yb mRNA levels varied directly with differences in RNA:DNA ratios whereas nuclear NF-Yb protein levels reflected DNA content, nuclear NF-Yb protein levels were likely regulated translationally or posttranslationally.

One indicator of protein synthesis rates is the number of ribosomes engaged on an mRNA (Hershey et al., 1986). As an indication of whether levels of NF-Yb protein escape cell size regulation translationally or posttranslationally, we measured the polysomal distribution of NF-Yb mRNA in a large-celled (liver, RNA:DNA = 4.7) and a small-celled (testis, RNA:DNA = 1.1) tissue (Fig. 6 *B*). If NF-Yb were translationally regulated, one would predict the mRNA to exhibit, on average, one fourth the number of ribosomes per message in liver as compared to testis (i.e., liver NF-Yb mRNA distributed into fractions much nearer the top of the gradient than that in testis). In contrast, only a minor fraction of the NF-Yb mRNA in both liver and testis was detected in fractions 1 and 2 (nonpolysomal fractions). NF-Yb mRNA was found in roughly the same distribution in liver and testis gradients, which suggests that the translational efficiency of NF-Yb was similar in both tissues. Our data suggest that liver synthesizes more NF-Yb protein per genome-equivalent than testis; however, nuclei from both tissues accumulate a similar steady-state number of NF-Yb protein molecules per genome-equivalent (Fig. 5 *B*). Thus, NF-Yb appears to escape cell size regulation via a posttranslational mechanism (see Discussion).

Microscopic examination and measurements by Wilson and Leduc (1948) indicated that liver cells undergo a large increase in average size with age. Consistent with this, we find that RNA:DNA ratios in mouse liver increase several-fold during liver maturation (Fig. 8). Although the liver from newborn rodents exhibits both hepatic and hematopoietic functions, livers from both young and old animals are primarily composed of parenchymal hepatocytes (for review see Gumucio and Chianale, 1988). Consistent with this, we find that a 2-d-old mouse liver contains 780 μg DNA, which is equivalent to 2.3×10^8 diploid genome-equivalents, whereas published values indicate the same liver will contain 10^5–10^6 hematopoietic cells (Kincade and Moore, 1977), representing less than 1% of the total cell population. Thus, maturing liver provided a system wherein we could test predictions for cell size–dependent regulation of genes en-

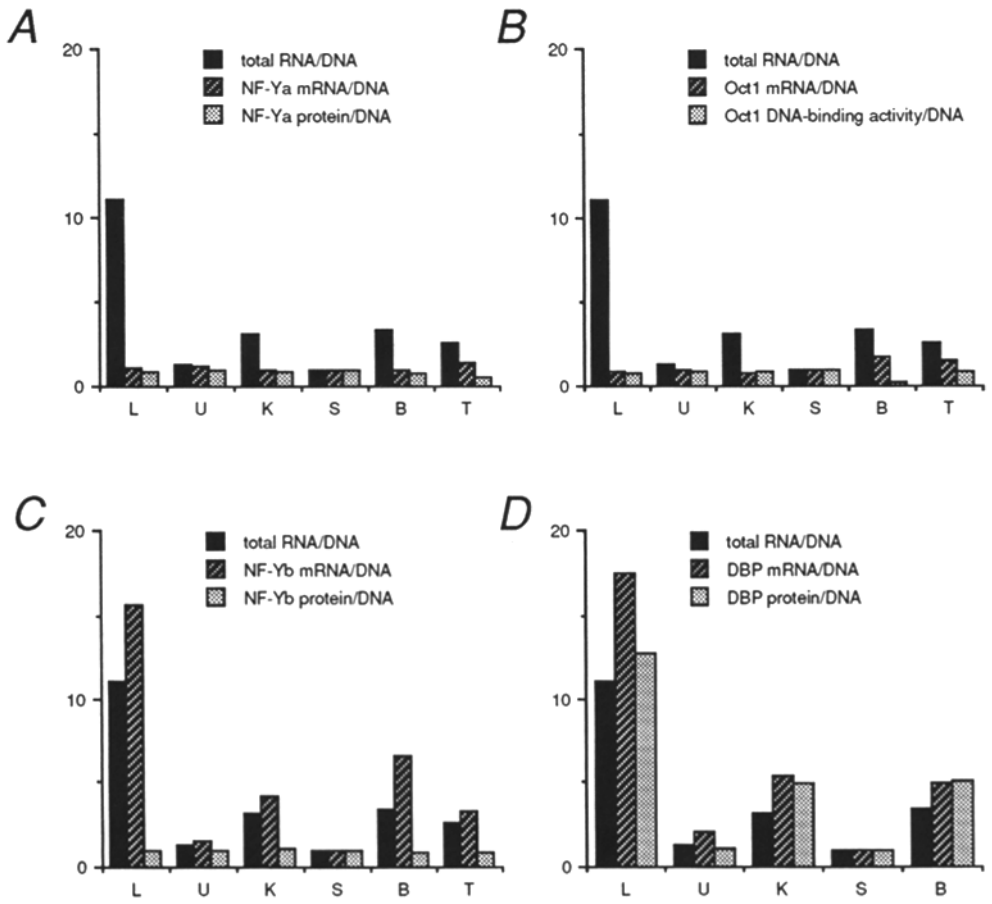


Figure 7. Quantitative tissue-specific differences in relative cellular levels of total RNA and of mRNAs and proteins for ubiquitous and tissue-enriched transcription factors. Signal intensities on autoradiograms were measured by laser densitometry and quantitatively related to macromolecule abundance by comparison to dilution curves. All values were normalized to DNA-equivalents; values for spleen (smallest average cell size) were arbitrarily set at 1.0 and other values are presented as relative to those for spleen.

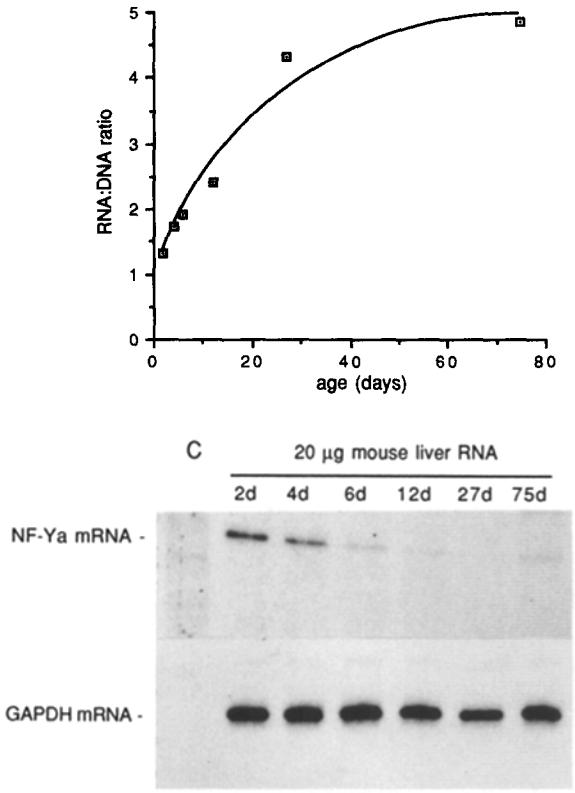


Figure 8. Changes in liver RNA:DNA ratio and in NF-Ya mRNA concentration during liver maturation. (*Upper panel*) Changes in liver RNA:DNA ratios. Total nucleic acid was prepared from the livers of mice of various ages (2, 4, 6, 12, 27, and 75 d) and RNA:DNA ratios were determined. (*Lower panel*) Expression of NF-Ya mRNA. RNA was prepared from an aliquot of each total nucleic acid sample above by sedimentation through cesium cushions. For NF-Ya RNase protections, 20 µg of RNA was used per reaction as described above. As a control, a parallel set of hybridizations was performed using 20 µg of RNA from each sample and a probe that spanned a 200-base EcoRI–AccI fragment of the rat GAPDH cDNA (10 fmol/reaction, specific activity 55 Ci/µmol).

coding ubiquitous proteins in a single organ type under conditions in which cell sizes, but not cell types, are substantially different. Concomitant with the increase in RNA:DNA ratios, levels of NF-Ya mRNA per total RNA decreased correspondingly. In contrast to NF-Ya mRNA, levels of the mRNA encoding glyceraldehyde phosphate dehydrogenase (GAPDH), a ubiquitous glycolytic enzyme (Lebherz and Rutter, 1967), represented the same proportion of total RNA in all samples (Fig. 8). In sum, the results presented above support the prediction that, for ubiquitous nuclear proteins to be expressed at the same level in all cells, the genes encoding these proteins must exhibit a mechanism to escape cell size regulation.

Characterization of a Transcription Factor That Is Subject to Cell Size Regulation. DBP, a liver-enriched transcription factor, was identified and its cDNA cloned by the ability of this protein to bind the "D-site" of the albumin promoter (Mueller et al., 1990). Expression of the *dbp* gene is maximal in adult animals during the evening hours. Western blots show that DBP protein is enriched in liver nuclei (Mueller et al., 1990; Wuarin and Schibler, 1990; see below); however, DBP protein can also be detected in lung, kidney, spleen, and brain nuclei, albeit at levels severalfold below that in liver (Fig. 9 *A*). All detectable DBP protein is nuclear (Wuarin and Schibler, 1990). Despite differences in nuclear DBP protein concentrations between tissues, DBP mRNA is similarly abundant in RNA harvested from liver, lung, kidney, spleen, and brain (Fig. 9 *A*; Mueller et al., 1990). Based on this observation, it was originally postulated that DBP protein accumulation may be posttranscriptionally controlled (Mueller et al., 1990).

Experiments were initiated to identify the causes for DBP enrichment in liver nuclei. The polysomal distributions of DBP mRNA in liver and lung were analyzed to compare translational efficiencies. Polysome gradients from both tissues exhibited a similar distribution of DBP mRNA, indicating that a similar number of ribosomes were engaged on each DBP mRNA molecule in both tissues (Fig. 9 *B*); DBP mRNA was not sequestered in the slowly sedimenting RNP fractions in lung (fractions 1 and 2). Our data suggest that DBP mRNA is translated with similar efficiencies in lung and liver.

Numerous cultured cell lines of hepatic and non-hepatic origin were analyzed in search of an in vitro system that would mimic the preferential accumulation of DBP in liver. Equal amounts of RNA from several rodent-derived cell lines and from rat liver were compared for endogenous levels of DBP mRNA. Surprisingly, all of the cell lines, whether of hepatic or fibroblastic origin, expressed relatively high levels of DBP nRNA/total RNA (10–20% of the maximal liver levels). Polysome analysis confirmed that DBP mRNA is engaged by ribosomes in "differentiated" hepatoma cells (H4.2 & C2Rev7), "dedifferentiated" hepatoma cells (H5), and fibroblastic L-cells; however none accumulated measurable levels of nuclear DBP protein (data not shown; hepatoma lines characterized in Deschatrette and Weiss, 1974; Deschatrette et al., 1980). Thus in terms of DBP accumulation, all of the cultured cells, including those that express liver-specific markers such as albumin mRNA, exhibited a non-liver phenotype. However, further examination revealed an important difference that could account for the liver enrichment of DBP protein: the cultured hepatoma cells, like the cells of lung, spleen, brain, and kidney, are much smaller than parenchymal liver cells.

How could cell size be responsible for the tissue-specific distribution of DBP? Because DBP mRNA levels are roughly proportional to rRNA levels, protein translated from a given number of DBP mRNA molecules should be concentrated into far fewer nuclei in large-celled tissues than that synthesized in small-celled tissues. The data are consistent with the model: translational efficiencies for DBP mRNA appeared to be similar in liver and lung (Fig. 9 *B*). Moreover, quantitative comparison of Western blot signals based on the standard curve containing dilutions of the liver nuclei indicates that liver nuclei contained more DBP protein molecules per genome-equivalent than lung, kidney, spleen, and brain nuclei by magnitudes which were proportional to the differences in RNA:DNA ratios between these organs (Fig. 9 *A*). Indeed, when Western blot and RNase protection assays are both normalized to equal genome-equivalents, our data show a very close correlation between total cellular RNA content, cellular DBP mRNA levels, and nuclear DBP protein levels (Fig. 7 *D*). This strongly suggests that tissue-

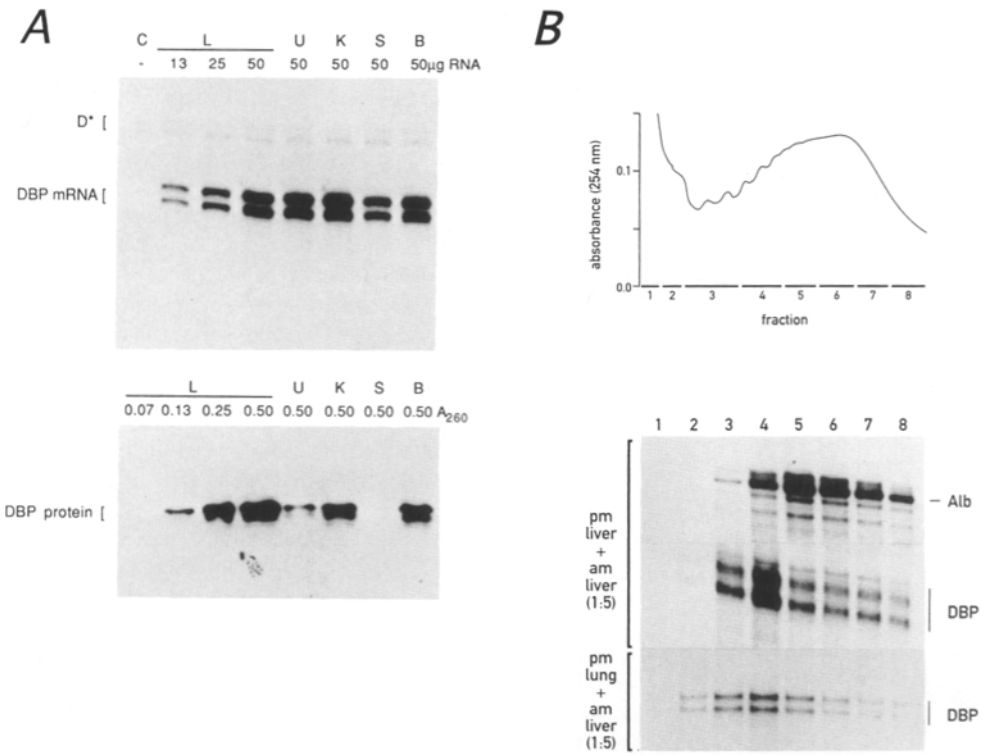


Figure 9. Expression of DBP in adult rat tissues. (*A*) DBP mRNA and protein levels. RNA was harvested from the indicated tissues from a rat killed at 5:30 pm (peak of circadian DBP mRNA expression, confirmed for all tissues studied, not shown); nuclei were prepared from tissues harvested at 8:30 pm (peak of circadian DBP protein expression, Lavery and Schibler, 1993). The upper panel shows an RNase protection assay using DBP probe and the indicated amounts of total RNA per sample supplemented with yeast RNA to 100 μg. All hybridizations contained 10 fmol of DBP probe (specific activity 55 Ci/μmol). The lower panel shows a Western blot using the indicated amounts of sonicated whole nuclei and a rabbit polyclonal antibody to DBP (Wuarin and Schibler, 1990) visualized by the ECL method. Dilutions of the liver samples allows quantitative comparison of signal intensities between tissue types for both DBP mRNA and protein. (*B*) Polysomal distribution of DBP mRNA. One gram of liver or lung harvested from a rat at 6:00 pm was homogenized in 10 ml each of a freshly thawed 50% rat liver cytoplasmic lysate prepared from an animal killed at 8:00 am (a control gradient confirmed that the morning extract did not contain significant levels of DBP mRNA; please see Materials and Methods for rationale) and polysome gradients were prepared with a 500-μl aliquot of each lysate. The upper panel shows the absorbance scan of a representative gradient; fraction 1 is the top of the tube. The lower panels shows albumin mRNA- and DBP mRNA-specific RNase protection signals from an aliquot (20% for DBP assays, 2% for albumin assays) of the RNA purified from each fraction of the indicated gradients supplemented with 50 μg (DBP) or 100 μg (albumin) yeast RNA. DBP hybridizations were as above; albumin samples were hybridized to 20 fmol probe (specific activity 5.4 Ci/μmol). Because lung is somewhat more difficult to homogenize than liver in nondenaturing buffers, quantitative comparisons of absolute mRNA levels between liver and lung cannot be made from the polysome data, but should be referred to *A*. Abbreviation: *D**, residual nondigested DBP probe.

specific nuclear DBP protein levels were determined by regulation of DBP mRNA levels. DBP mRNA levels, in turn, matched total RNA levels, suggesting that the *dbp* gene might be subject to cell size regulation. If this were the case, one would expect tissue-specific DBP mRNA levels, like tissue-specific total RNA levels, to be determined at a transcriptional level. Therefore, we tested this prediction.

Nuclear Control of mRNA Levels for NF-Ya, Oct1, and DBP. The levels of NF-Ya, Oct1, and DBP proteins generally corresponded to levels of their cognate mRNAs in the samples that we examined (see above). Therefore, the cell size–dependent regulation of the cognate genes must occur by regulation of either transcription rate or transcript stability. Transcription rates for the *nf-ya* and *oct1* genes have proven too low to be measured by nuclear run-on assays. For this reason, an assay for nuclear concentrations of NF-Ya, Oct1, and DBP mRNAs was used to further study the levels of regulation. The rationale behind this assay follows.

The differences between nuclear and whole cell concentrations of albumin mRNA (concentration taken as albumin mRNA per total RNA in the sample) in Fig. 10 was roughly 0.05 (the nuclear RNA lane contained 20-fold more input RNA than the whole cell RNA lane for the albumin mRNA assay). This shows that cytoplasmic contamination of the nuclear preparations was less than 5%. For Oct1, NF-Ya, and DBP mRNAs, the ratios of nuclear:whole cell concentrations were all roughly three (nuclear and whole cell lanes contained equal amounts of input RNA for Oct1, NF-Ya, and DBP mRNA assays). Thus the maximal error due to cytoplasmic contamination (+0.05) was negligible. The kinetics of the circadian expression pattern of DBP mRNA indicate that its halflife is on the order of 1–2 h (for mathematical model, see Wuarin et al. [1992]). Because NF-Ya and Oct1 mRNA showed nuclear/cytoplasmic distributions similar to that for DBP mRNA, all three transcripts likely have similarly short halflives. For unstable mRNAs such as these, a relatively large fraction of the lifetime of the mRNA is expected to be spent in the nucleus during synthesis and processing (Wuarin and Schibler, 1994). The high nuclear:cytoplasmic ratio measured for these mRNAs is consistent with this prediction.

Having established that the nuclear preparations were not significantly contaminated by cytoplasmic RNA, we asked whether the relative expression patterns seen for Oct1, NF-Ya, and DBP mRNAs in whole tissues are determined in the nucleus (Fig. 10). Again, in interpreting the data it is impor-

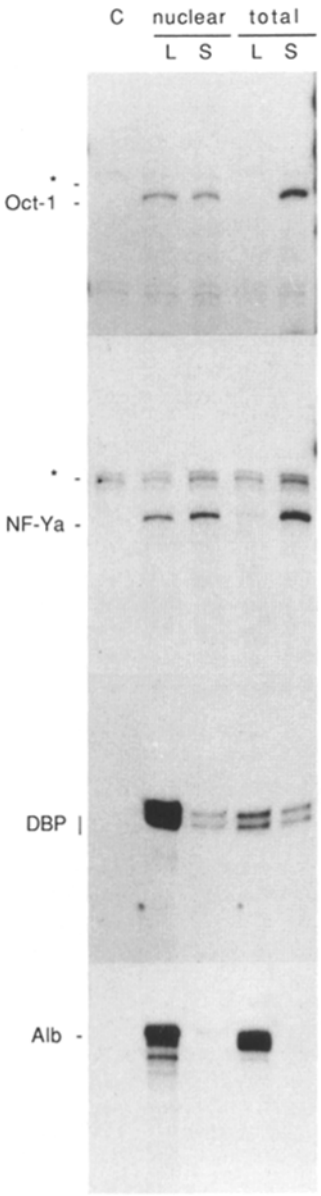


Figure 10. Nuclear and total concentrations of Oct-1, NF-Ya, DBP, and Alb mRNAs in liver and spleen. Total RNA was prepared from liver and spleen of mice and rats killed at 5:30 pm (circadian peak of DBP mRNA levels). Nuclei were prepared from liver and spleen of mice and rats killed at 3:00 pm (circadian peak of DBP transcription, Lavery and Schibler, 1993) and nuclear RNA was purified through cesium cushions. RNase protection assays were as above except that for albumin, the probe specific activity was 55 Ci/μmol. For Oct-1 mRNA determinations, hybridizations contained 25 μg per lane of nuclear or total mouse RNA; for NF-Ya, 50 μg of nuclear or total mouse RNA was used; for DBP, 50 μg of total rat RNA was used; and for albumin mRNA levels, 10 μg of nuclear or 0.5 μg of total rat RNA were used. In all hybridizations, samples were supplemented with yeast RNA to 50 μg. Control lanes contained probe plus 65 μg of yeast RNA. Asterisks denote residual nondigested probe.

tant to consider the different RNA:DNA ratios between liver and spleen. Signals in the nuclear lanes represent nuclear quantities of transcripts in liver and spleen. In contrast, the whole cell RNA lanes reflect the concentrations of Octl, NF-Ya, or DBP mRNA, and must be corrected for RNA:DNA ratio differences between the tissue types (11-fold) to estimate per-genome mRNA levels. The results show that liver and spleen have a similar number of nuclear Octl and NF-Ya mRNA molecules per genome-equivalent, and similar numbers of total Octl and NF-Ya mRNA molecules per genome-equivalent (Figs. 5, 6, and 10). Levels of nuclear DBP mRNA per DNA also corresponded to levels of whole cell DBP mRNA per DNA in both tissues (Figs. 9 and 10), and differed between tissues by a magnitude reflecting the RNA:DNA ratio differences. The correlation between nuclear and whole cell mRNA levels strongly suggests that cellular levels of these mRNAs were determined in the nucleus. Our results are consistent with regulation of all three genes at a transcriptional level. Therefore, we conclude that the *dbp* gene is likely subject to cell size regulation, whereas the *nf-ya* and *octl* genes likely use a transcriptional mechanism to escape cell size regulation.

Discussion

Cell Size Regulation

Different cell types within an organism can vary greatly in size. In comparing tissues from adult rodents, we found that differences in cell size generally corresponded to differences in cellular RNA content. Hence, we entitle the mechanisms controlling the cell type–specific accumulation of RNA cell size regulation. We present data showing a tight correlation between transcription rates and RNA:DNA ratios in liver, lung, kidney, spleen, brain, and thymus. Therefore, in these tissues, the cellular RNA levels, and thus the RNA:DNA ratios, are likely to be primarily determined by transcriptional mechanisms.

Importantly, differences in total transcriptional activity between liver and thymus or spleen (6–26-fold, Fig. 3) were similar to differences in pol II–specific transcriptional activity between these tissues (10–11-fold, Table II). As pol II activity represents only ~30% of the total RNA polymerase activity (Roeder, 1974), regulation of pol II alone could not account for the difference in overall transcription rates. Rather, the activities of pol I and pol III, in combination, must also be cell size dependent. For pol II, our data suggest that enzyme activities per unit pol II protein differ between cell types (Fig. 4). For pol I and pol III, it remains untested whether protein levels are regulated. It might be interesting in this regard that the genes encoding the subunits of all three polymerases are likely to be transcribed by pol II. Thus, it is feasible that regulation of pol II activity could regulate levels of the other polymerases. At this point we remain far from understanding the precise molecular mechanisms that determine the cell size–dependent transcription rates.

Little is known about the genetic processes that might determine how much cytoplasmic material a cell will accumulate, and, indeed, we are aware of no cell size mutants in mammals. Nevertheless, in an insightful study, Dobzhansky (1929) compared cell sizes between wild-type and miniature *Drosophila* mutants. This work showed that individual genes can affect, and therefore potentially regulate, cell size. A more recent molecular analysis of one miniature mutant mapped the defect to a gene encoding a ribosomal protein (Kongsuwan et al., 1985). The observation that the miniature phenotype can be caused by a mutation in a ribosomal protein suggests that differences in the translational efficiency in different cells can lead to different cell sizes. In contrast, we found no significant difference in translational efficiency per unit RNA between liver and spleen cells despite a large difference in cytoplasmic:nuclear volume ratios (Fig. 3). However, theoretically, both differences in the ability of ribosomes to translate mRNA and differences in the amount of mRNA and ribosomes which are available for translation should have the same ultimate effect: to change the rate of cellular protein synthesis. Thus, the miniature mutants indicate that cell size could be regulated by altering rates of protein synthesis, whereas our study extends this possibility by suggesting that overall rates of protein synthesis, and thus

cell sizes, are likely regulated at a transcriptional level in several rodent tissues.

Having cell size differences in metazoan organisms may impose constraints on gene regulation that will not necessarily exist in single-celled organisms. In multicellular organisms, wherein the steady-state cytoplasmic content of individual postreplicative somatic cells can vary by more than an order of magnitude, at least two classes of house-keeping genes can be distinguished. The first class encodes components whose numbers are proportional to cell size, such as ribosomes, tRNAs, structural cytoplasmic proteins, and translation factors. The second class encodes products whose numbers match cellular DNA content, such as histones and ubiquitous transcription factors. As discussed below, the consequences of cell size regulation are particularly intriguing for the accumulation of nuclear proteins, such as transcription factors.

Cell Size and Gene Regulation

Although cell size, like all heritable characteristics, is no doubt genetically regulated (Dobzhansky, 1929), the converse possibility, that specific gene expression patterns might be regulated by cell size, has been explored in few situations. Classical work on fission yeast showed that cell size feeds back on cell cycle progression through a mechanism that regulates the expression of specific genes in response to volume (Fantes, 1977; Fantes and Nurse, 1977). Subsequent studies on metazoans suggested that similar mechanisms likely contribute universally to patterns of replication-specific gene expression (Baserga, 1984; Edgar et al., 1986). Also, asymmetric cell divisions which lead to daughter cells of different sizes have been correlated to genetic cell fate determination (for review see Horvitz and Herskowitz, 1992; and see Kirk et al., 1993, for an intriguing analysis). However, to our knowledge, no one has investigated whether the vast differences in cell size between postreplicative somatic cell types might influence tissue-specific gene expression in metazoans.

We demonstrate that cell size regulation may cause DBP protein to accumulate to higher levels in the nuclei of large cells, such as parenchymal hepatocytes. Conversely, several ubiquitous transcription factors apparently require mechanisms to ensure cell size–independent accumulation. Fig. 11 shows a schematic representation of the cell size–dependent expression patterns for the three classes of nuclear proteins that we studied. In the model, NF-Ya and Oct1 use transcriptional mechanisms to match protein levels to DNA content.

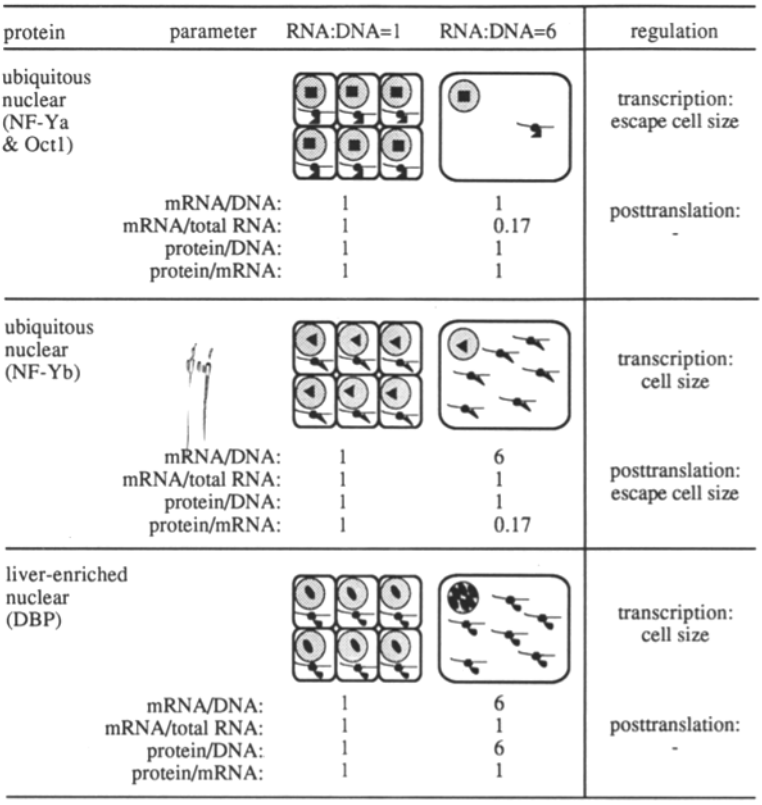


Figure 11. Cell size–dependent regulation of nuclear protein accumulation. Parameters that determine the nuclear concentrations of NF-Ya, Oct1, NF-Yb, and DBP are portrayed in hypothetical cells exhibiting sixfold differences in RNA:DNA ratios. In the diagrams, an equal tissue-mass of each cell type (i.e., equal RNA-equivalents) is shown to emphasize the difference in RNA:DNA ratios and the distinction between components expressed at equal numbers or equal concentrations in the cells or compartments. Nuclei are shown as shaded circles; nuclear proteins are shown as dark squares (*NF-Ya* or *Oct1*), triangles (*NF-Yb*), or ellipses (*DBP*) in the nuclei. mRNA levels are shown as cytoplasmic monosomes with a nascent polypeptide to imply mRNA-dependent synthesis rates. Although only one monosome is shown for NF-Ya in the large cell, the total number of ribosomes (and total RNA) will be the same in all of the large cells (proportional to RNA:DNA ratios). In calculating protein levels, only the mature nuclear species, not the nascent polypeptides, are considered. The values below each diagram correspond to relative amounts of the specific mRNAs or proteins as normalized to either cellular DNA content, total RNA content, or levels of the specific mRNA, as indicated. The upper panel, for NF-Ya and Oct1, shows that these genes are regulated such that both cell types contain an equal number of mRNAs for these proteins, and thus an equal number of the cognate proteins per nucleus. In the middle panel, NF-Yb mRNA levels are expressed at equal concentrations in all cells. Thus, the large cell has sixfold more NF-Yb mRNA molecules than any one of the small cells. Protein levels are posttranslationally matched to DNA content. In the lower panel, DBP mRNA is also expressed at the same concentration in both cell types. However, as DBP protein is not matched to DNA content, the large cell accumulates more nuclear DBP protein. For simplicity, the model shows no posttranslational control of NF-Ya, Oct1, or DBP levels. Although this is consistent with our data, we do not wish to imply that other levels of regulation do not exist under conditions that we did not test. It is noteworthy that the ubiquitous proteins are the same concentration in all nuclei (protein/DNA), but different concentrations in the tissues (protein/tissue mass). DBP protein is the same concentration in both tissues, but different concentrations between nuclei types. The concentrations of NF-Ya and Oct1 mRNAs differ between tissues; the concentrations of NF-Yb and DBP mRNAs are the same in both tissues.

In contrast, transcription of the *nf-yb* gene and synthesis of NF-Yb protein are cell size–dependent; protein levels are matched to nuclear content by a posttranslational mechanism. Finally, transcription of the *dbp* gene and synthesis of DBP protein are also cell size–dependent; however unlike NF-Yb, DBP protein is not matched to DNA content. Therefore, DBP preferentially accumulates in the nuclei of cells with large RNA:DNA ratios.

Liver nuclei are larger than thymus or spleen nuclei (Figs. 1 and 3). Thus, one may ask to what extent differences in nuclear volumes could influence the interpretation of our data. Since our conclusions are based on data normalized to DNA content, the interpretations are not affected by differences in ploidy. Also, nuclear transcription factors are probably not free in solution. Indeed, when liver nuclei are disrupted by sonication in large volumes of physiological saline with nonionic detergent and chromatin fragments are sedimented by low-speed centrifugation, DBP protein quantitatively sediments with the chromatin (not shown). Excess recombinant DBP, if added to nuclei, associates quantitatively with the chromatin (not shown), suggesting that stable binding sites for DBP in chromatin are not saturated. This renders unlikely the possibility that, while in cells, nuclei contain additional DBP which is free in solution and is lost during isolation of nuclei. Thus for transcription factors, we suspect that the chemical concentrations of the factors in nuclear solute is probably a less important parameter than the absolute amount of factor per DNA.

Escape from Cell Size Regulation. The term "escape from cell size regulation" implies that the expression of most genes would be affected by cell size regulation unless they have specific signals to override the effect. Indeed, we show that overall RNA synthesis rates and total mRNA levels are cell size dependent, which suggests that cell size regulation is a fairly consummate process. Moreover, the *nf-yb* gene exhibited cell size–dependent mRNA levels and subsequent posttranslational regulation to achieve cell size–independent protein levels. Thus, we have the impression that genes encoding ubiquitous cytoplasmic molecules are subject to the "default" regulatory pathway and those encoding ubiquitous nuclear products actively escape this control.

Levels of NF-Ya and Oct1 mRNAs appear to escape cell size regulation by a nuclear mechanism (Fig. 10). The observation that amounts of NF-Ya and Oct1 transcripts in nuclei roughly matched amounts of NF-Y and Oct1 DNA-binding activity could readily be explained by a direct feedback inhibition loop. In the simplest form of this model, when the level of a ubiquitous transcription factor reached a certain threshold level, it would downregulate its own gene and possibly genes encoding other ubiquitous nuclear products as well. This would match transcription rates on these genes to DNA content and make expression independent of cell size regulation.

Our data suggest that liver cells, on average, synthesized NF-Yb protein at several times the per-genome rate of testis cells; yet nuclei from both tissue types exhibited the same amount of NF-Yb protein per DNA. This indicates that nuclear levels of this protein were likely determined posttranslationally. Such an expression pattern could arise by regulating NF-Yb nuclear localization, NF-Yb protein stability, or both. The sequence of NF-Yb reveals no obvious nuclear localization signal (Boulikas, 1993); however, we have been unable to detect NF-Yb protein accumulation in liver cytoplasm by immunoprecipitation and Western blot analyses (not shown). Thus, it appears likely that excess NF-Yb protein is degraded in large cells. Because NF-Y is an obligatory heteromer containing NF-Ya and NF-Yb (Chodosh et al., 1988*a*,*b*; Forsburg and Guarente, 1989; Hatamochi et al., 1988; Hooft van Huijsduijnen et al., 1990; Maity et al., 1992; Mantovani et al., 1992) and because NF-Ya protein exhibits an independent mechanism of escaping cell size regulation (see above), it is possible that NF-Yb protein levels are matched to NF-Ya protein levels by a mechanism that degrades NF-Yb monomer preferentially, analogous to the system which makes stability of IκB dependent on levels of NFκB (Brown et al., 1993; Scott et al., 1993; Sun et al., 1993).

Cell Size May Contribute to Tissue-Specific Transcription Patterns. A fundamental question in metazoan cell biology asks how clonally derived genetically identical cells can establish and maintain different phenotypes. Tissue-specific transcription has been shown to play important roles in cell type specification, but what makes certain transcription factors tissue enriched? In some cases, direct regulation by another tissue-enriched transcription factor has been demonstrated. For example HNF-1 (Courtois et al., 1988), a transcription factor that appears to play a role in the liver-specific expression of the albumin gene (Cereghini et al., 1987; Lichtsteiner and Schibler, 1989), is itself highly enriched in liver nuclei as a result of a transcriptional control mechanism in which HNF-4 protein, another liver-enriched transcription factor, appears to play a predominant role (Kuo et al., 1992; Tian and Schibler, 1991). In other cases, tissue-specific transcription factors have been shown to autoactivate transcription of their own gene via an autoregulatory loop, and thus maintain their own tissue specificity (e.g., Thayer et al., 1989; Way and Chalfie, 1989).

The cell size–dependent expression pattern for DBP protein that we outline in this paper provides an interesting alternative mechanism by which a transcription factor can become tissue enriched. Our data on DBP accumulation require neither an upstream liver-specific transcription factor nor an autoregulatory loop to explain the liver-enriched expression pattern. Rather, the expression pattern for the *dbp* gene appears to be dependent on signals that regulate cellular RNA:DNA ratios and, as a consequence, cell size. We suggest that nuclei of large cells such as parenchymal hepatocytes transcribe the *dbp* gene, like the genes encoding ubiquitous cytoplasmic molecules, at a higher rate than the nuclei of smaller cells. As DBP protein is exclusively localized to the nucleus, it becomes enriched in the nuclei of large cells as compared to the nuclei of small cells.

Cell Size Must Be Considered for Molecular Comparisons between Cell Types. To appreciate the practical importance of cell size regulation for interpreting data on gene expression, one must critically evaluate the denominator for each measurement. The purpose of measuring specific transcription rates or mRNA levels is to gain insights into the mechanisms regulating expression of the cognate genes. It is striking in this regard that these measurements are almost never normalized to equal gene-equivalents. Northern blot analyses and nuclease protection assays are invariably normalized to bulk RNA (or poly-A+ RNA). Therefore, such assays can give no indication of the relative activities of a

specific gene between cell or tissue types unless the total RNA contents per genome-equivalent are known and considered for all samples. As seen above, DBP mRNA represents the same fraction of total RNA in samples from liver and spleen, yet because the cell sizes differ, liver accumulates 11-fold more DBP mRNA and protein molecules per gene copy than spleen. Conversely, the fraction of total RNA represented by NF-Ya or Octl mRNAs are 11-fold higher in spleen RNA as compared to liver RNA, yet, as a result of cell size differences, these mRNAs and their cognate proteins accumulate to a very similar number in the two cell types. Cell size also has to be considered for in situ hybridization analyses, in particular for mRNAs encoding nuclear proteins. As suggested by the diagram in Fig. 11, in situ hybridization with probes for DBP mRNA would show uniform signals in large and small cells. Conversely, in situ hybridization for NF-Ya or Octl mRNAs would reveal much stronger signals in small than in large cells. Unless one is conscious of cell size differences, such observations might invite the entirely false conclusion that DBP mRNA (and perhaps protein) is equally abundant in all cells and NF-Ya mRNA (and perhaps protein) levels are cell type-specific.

Finally, overall transcription rates are cell size dependent (Table II, Fig. 3). In nuclear run-on transcription, overall incorporation is greater in cells with greater RNA:DNA ratios. As a result, nuclear run-on assays normalized either to input radioactivity in the hybridization or to specific signals obtained with genes encoding ubiquitous cytoplasmic molecules, such as the commonly used GAPDH or tRNA genes, do not compare an equal number of gene copies, and therefore do not reflect relative per-gene RNA polymerase densities between nuclei of different cell types. To measure per-gene transcription rates, the hybridization signals per input radioactivity must be corrected for differences in the ratio of total incorporated radioactivity per DNA-equivalent.

We suspect that a simple reevaluation of existing data on cell type–specific gene expression patterns, after normalizing all values to equal genome-equivalents as the common denominator, may provide unexpected new insights into gene regulation.

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