Post-transcriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues

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ABSTRACT

We have isolated and identified cDNA clones containing part of the coding sequence for rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, E.C. 1.2.1.12). By using one of these clones as a probe, we have shown that: i) the abundance of GAPDH mRNA is different in various tissues of the adult rat and in good correlation with the abundance of the enzyme; ii) the transcription rates are quite similar in all tissues tested. We therefore conclude that the tissue-specific differential GAPDH gene expression is regulated by adjusting the abundance of its mRNA at the post-transcriptional level.

INTRODUCTION

While a considerable wealth of information has been accumulated about gene regulation concerning specialized functions such as those involved in hormonal response or tissue-specific gene expression, little is known about the way house-keeping functions are regulated. Our laboratory is currently interested in the structure and expression of genes coding for enzymes involved in major metabolic pathways and especially glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, E.C.1.2.1.12) which is a key enzyme of glycolysis present in every tissue, albeit at quite different levels (1).

It is made up of four identical polypeptide chains whose aminoacid sequence has been determined in a wide variety of organisms (2). Despite the presence of an amazingly high number (> 200) of pseudogenes in rat and mouse (Piechaczyk et al., submitted for publication; Hanauer and Mandel, personal communication), it is encoded by a single functional gene in all species, including rat, studied so far (3-8).

The ubiquity of GAPDH allows to explore the expression of its gene in quite different cellular contexts. To this aim, we have constructed and identified a cDNA clone encoding part of rat GAPDH and used it to analyse the expression of GAPDH gene in various rat tissues (muscle, liver brain,

kidney, heart and testis). This analysis involved steady-state measurements of GAPDH protein, of its messenger and of nascent nuclear transcripts. The results of this study clearly show that the enzyme is differentially expressed from one tissue to another and its abundance is correlated with the abundance of its message. However the GAPDH gene appears to be transcribed at the same rate in all the analysed tissues. This indicates that the main regulation occurs at the post-transcriptional level.

MATERIALS AND METHODS

Animals

For each experiment, four 39 days old inbred Wistar rats were perfused with physiological saline and their organs pooled.

RNA preparation, Northern blots and dot-spots

Total RNA was extracted from rat tissues by the LiCl-urea procedure of Auffray and Rougeon (9). Poly (A)⁺ RNA was selected through two cycles of oligo (dT)-cellulose chromatography as described (10).

Northern blots of RNA samples electrophoresed on denaturing 1.2% agarose gels were made on nitrocellulose membranes as described by Thomas (11). RNA and M13 ssDNA samples for dot-spots were denatured for 10 min. at 60°C in 1M formaldehyde in a volume of 50 µl. Solutions were then adjusted to 1 ml with 2X SSC (1X SSC= 0.15M NaCl, 0.015M Na Citrate) and immediately filtered through a 20X SSC prewetted nitrocellulose membrane by using the BRL Hybridot system. DNA samples were denatured by boiling for 10 min. in 0.3M NaOH at a concentration of 50 µg/ml and chilled in ice. They were neutralized by 1ml of cold 2M ammonium acetate. Fixation of DNA onto nitrocellulose membranes prewetted with ammonium acetate was done as above.

Construction and identification of a GAPDH cDNA clone from rat C6 glioma cells.

Double-stranded cDNA was prepared by the double-tailing procedure (12,13) from poly(A)⁺ RNA from nearly confluent C6 cells (kindly obtained from Dr. A. Tixier-Vidal) and annealed to oligo-dG tailed pBR322 plasmid (14) (New England Nuclear) in a 1:1 molar ratio. DP50 (dap⁻, thy⁻) E. coli cells rendered competent by CaCl₂ treatment (15) were transfected with the recombinant plasmids, plated at high density and screened on Gene-Screen membranes (New England Nuclear) as described by Hanahan and Meselson (16) using the purified insert from a partial GAPDH cDNA clone from chicken muscle kindly supplied by Dr. A.M MacLeod (17). 1ng of ds-cDNA currently yielded 100-150 clones. Recombinant plasmids were prepared using the alkaline method

of Mc Master et al. (18). The DNA insert was excised with PstI and labeled with (^{32}P) α dideoxyadenosine triphosphate and deoxynucleotidyl terminal transferase (19). After secondary cleavage with AluI, singly labeled DNA subfragments were sequenced by the chemical procedure of Maxam and Gilbert (20). Alternatively, the same unlabeled subfragments were cloned and sequenced in M13mp11 (21,22). In both cases, reaction products were separated on either 15 % or 6 % 0.4 mm thick polyacrylamide gels (23) containing 8M urea prepared with or without a buffer gradient (24).

All experiments were carried out in compliance with the guidelines of the French National Committee for recombinant DNA.

Western blots

Cytoplasmic extracts were prepared by homogenizing perfused organs with a Kontes homogenizer in 10mM Tris-HCl pH 7.4, 10mM NaCl, 10mM MgCl_2 , 0.5% NP40 at 0°C. Nuclei were spun down for 10 min. at 2,500 rpm and supernatants were directly used for immunological detection on western blots (25) using an anti-human GAPDH antibody raised in rabbit. Protein concentration was assayed according to Witaker and Granum (26).

In vitro translation

The reticulocyte lysate of Amersham was used under the conditions specified by the supplier. Immunoprecipitation of in vitro synthesized GAPDH was done by the Protein A-Sepharose method of Mota et al. (27).

Radioactive labelling and hybridization conditions

Radioactive labelling of plasmids or purified inserts was done with the nick translation kit of Amersham as specified by the supplier. After overnight prehybridization in 50% formamide, 0.75M NaCl, 50mM Na phosphate pH7, 0.1% polyvinylpyrrolidone, 10% dextran sulfate, 0.1% Ficoll, 0.5% SDS, hybridizations were carried out for 24 hours with 1ng/ml of nick-translated probe ($1\text{--}2 \times 10^8$ dpm/ μg). Three 10 min. washings were done in 2X SSC, 0.5% sodium dodecylsulfate at room temperature. Stringent washings were done in 0.2X SSC, 0.5% SDS at 60°C for a period of 1 to 2 hours.

Elongation of nascent chains in isolated nuclei

Nuclei were purified from fresh perfused organs and incubated exactly as described by Schibler et al. (28). Nuclei from muscle and heart were largely contaminated by actin filaments with no subsequent interference on UTP incorporation into nascent transcripts. Incorporations were about 0.5 to 1×10^5 dpm per μg of input DNA. Filters dot-spotted with 5 μg of each plasmid DNA were processed as above except for 3 days of prehybridization, 4 days of hybridization and the absence of dextran sulfate (28).

Figure 1: Sequencing strategy.

The insert from pRGAPDH-1 clone was excised by PstI, recleaved by AluI and sequenced in the directions indicated by arrows by either the Maxam-Gilbert (M.G.) or M13 procedures as described under Materials and Methods. Shaded areas marked G_n and C_n refer to the homopolymeric extensions used for cDNA cloning.

Autoradiography

Autoradiographs were performed on Kodak XAR film with a Quanta III Dupont Cronex intensifying screen at -70°C .

RESULTS

Characterization of a rat GAPDH cDNA clone from C6 glioma cells.

8,000 ampicillin-sensitive, tetracycline-resistant clones were screened at high density using the (^{32}P) nick-translated pGPD1 chicken GAPDH cDNA probe (17) under low stringency conditions of hybridization (30% formamide, 0.75M NaCl, 37°C). 20 positive clones were replated at low density and sequencing of the insert from one clone, pRGAPDH-1, was obtained by a combination of chemical and enzymatic methods according to the strategy outlined in Figure 1. The resulting nucleotide sequence is shown in Figure 2 together with the derived aminoacid sequence and compared with the corresponding chicken sequence (29-31). Both nucleotide and aminoacid sequences unambiguously demonstrate that this clone does encode GAPDH.

The cDNA insert of pRGAPDH-1 is entirely contained within the coding sequence close to its COOH terminus (from aminoacid 261 to 324 with reference to the chicken sequence) but includes neither end of the messenger despite the double-tailing method used (12,13). This clone was used without further investigating possible explanations for this peculiarity (internal tailing or incomplete second strand synthesis).

Various rat tissues contain different levels of GAPDH

GAPDH being known to be essentially cytoplasmic (1), cytoplasmic extracts of muscle, heart, testis, liver, brain and kidney from adult Wistar rats were electrophoresed on SDS-acrylamide gels, transferred to nitrocellulose and subjected to immunological detection (25) by rabbit

(a)	TG	AAG	CAG	GCG	CCC	GAG	GGC	CCA	CTA	AAG	GGC	ATC	CTG	GGC	TAC	ACT	GAG
(b)		GCT	T	T	T	G	C	G						A	A		A
(a)		K	Q	A	A	E	G	P	L	K	G	I	L	G	Y	T	E
(b)		A				D											

(a)	GAC	CAG	GTT	GTC	TCC	TGT	GAC	TTC	AAC	AGC	AAC	TCC	CAT	TCC	TCC	ACC	TTC
(b)									T	G	T	G	AG				
(a)	D	Q	V	V	S	C	D	F	N	S	N	S	H	S	S	T	F
(b)									G	D							

(a)	GAT	GCT	GGG	GCT	GGC	ATT	GCT	CTC	AAT	GAC	AAC	ATT	GTG	AAG	CTC	ATT	TCT
(b)		G	T				A	G			C	T	T	C		T	G
(a)	D	A	G	A	G	I	A	L	N	D	N	I	V	K	L	I	S
(b)											F					V	

(a)	TGG	TAT	GAC	AAT	GAA	TAT	GGC	TAC	AGC	AAC	AGG	GTG	GTG	GA			
(b)					G	T	A				C	T	T				
(a)	W	Y	D	N	E	Y	G	Y	S	N	R	V	V				
(b)							F										

Figure 2: Nucleotide and aminoacid sequence comparison of rat (a)

pRGAPDH-1 and the corresponding part of chicken (b) pGPD-1.

The pGPD-1 sequence shown here was previously determined (29-31). Only those nucleotides or aminoacids which are different in chicken as compared to rat are shown. The GAPDH aminoacid sequence presented spans from residue 261 to 324 with reference to the chicken sequence.

anti-GAPDH antibodies (Figure 3A). After densitometric scanning of these western blots, the relative GAPDH content of each tissue was expressed with reference to the lowest level observed in testis (Table I, column A). These data show that GAPDH content follows this order: muscle > heart > liver, kidney, brain > testis, in good agreement with the situation derived in man and rat (1) from enzymatic determinations, except for testis in which it was not determined.

GAPDH levels are correlated with the abundance of its mRNA

In a first set of experiments, nick-translated pRGAPDH-1 DNA was used to probe northern blots of total RNA extracted from different tissues. Results presented in Figure 3B show that GAPDH mRNA is expressed in every tissue as a molecule of similar mobility, suggesting that these messages are not qualitatively different. They also show that the different rat tissues contain very different amounts of total GAPDH mRNA but in clear correlation with the amount of GAPDH protein.

In order to obtain an absolute measurement of the proportion of GAPDH

Figure 3: Comparative levels of GAPDH protein and mRNA in rat tissues.

A) Immunological detection on western blots: 50µg of total cytoplasmic proteins from each tissue were run on 12.5% polyacrylamide gels, blotted and reacted with anti-GAPDH antibody as described in Materials and Methods. B) and C) Northern blots of total (20µg) and poly(A)⁺ RNA (1µg) were probed with pRGAPDH-1 as described in Materials and Methods.

mRNA in total RNA, various quantities of total RNA from the different tissues were dot-spotted on nitrocellulose and hybridized with the same probe. Hybridization signals (not shown) were quantitated by densitometry scanning using a single-stranded M13-GAPDH recombinant as a reference and expressed as weight percentages of GAPDH mRNA (Table 2, column A).

Relative values of GAPDH mRNA in the different tissues have been determined from densitometer tracings of both northern and dot-blots (Figure 4, left panel) and found to be quite similar for the two techniques. Altogether they show a good correlation between the relative levels of GAPDH and its message (Table 1, columns B and C).

Most GAPDH mRNA is polyadenylated in all rat tissues.

The criterion which defines polyadenylation being entirely operational, i.e. the fraction of total RNA which is retained on oligo(dT)-cellulose, we first measured the minimum length of poly(A) necessary for binding by running radioactive oligo(A) fragments on such a column and could determine a cut-off value around 10-15 A residues. Any RNA molecule bearing a smaller poly(A) tail would therefore be scored as poly(A)⁻.

The comparison of northern blots of poly(A)⁺ RNA (Figure 3C) and total RNA (Figure 3B), indicates that most, if not all GAPDH RNA is polyadenylated in the various tissues. However the proportion of total poly(A)⁺ RNA varying from one tissue to another and being rather difficult to determine precisely, quantitations were done in another way. Known quantities of total RNA from

TABLE 1: Relative levels of GAPDH protein, mRNA and in vitro runoff nuclear transcripts in various rat tissues.

	A	B	C	D
	GAPDH proteins	GAPDH mRNA in total RNA	GAPDH mRNA in poly(A) ⁺ RNA	Transcription rate
MUSCLE	19	24.4	20.9	1.06
HEART	10	11.4	11.3	1.03
LIVER	5.5	4.2	3	0.73
BRAIN	5.5	5.6	5	0.80
KIDNEY	5.5	4.3	4.5	0.72
TESTIS	1	1	1	1

Column A) : Autoradiographs of western blots from Figure 3A were scanned and the areas of GAPDH peaks were normalized relatively to the value for testis taken arbitrarily as 1. Column B) : Figures of this column are averages of two sets of values. The first set is derived from autoradiographs of northern blots from Figure 3B scanned and normalized as in A). The second set, actually quite comparable to the first one, comes from dot-blot data from Table 2, column A, normalized in the same way. Column C) : Average figures derived as in B) from northern blots of Figure 3C and dot-blot data from Table 2 column B. Column D) : Dots of GAPDH transcripts from two experiments as in Figure 5 were scanned, normalized as in A) and averaged.

TABLE 2 : Proportions of GAPDH mRNA, expressed as weight percentages, in total, poly(A)⁺ RNA and translatable RNA from various rat tissues.

	A	B	C
	GAPDH mRNA in total RNA	GAPDH mRNA in poly(A) ⁺ RNA	In vitro translated GAPDH
MUSCLE	0.2 %	3.6 %	4.3 %
HEART	0.1 %	2 %	2 %
LIVER	0.029 %	0.5 %	0.9 %
BRAIN	0.034 %	0.8 %	1.5 %
KIDNEY	0.028 %	0.8 %	1 %
TESTIS	0.008 %	0.16 %	0.5 %

Column A) : A scale of total RNA dots and a reference scale of dots containing coding strand GAPDH DNA (as an M13 clone carrying the pRGAPDH-1 insert) were probed in parallel with nick-translated pRGAPDH-1. The proportion of GAPDH RNA present in an RNA dot was estimated by comparison with the reference scale. Column B) : Same as in A) except that poly(A)⁺ RNA was used instead of total RNA. Column C) : Figures in this column represent the fraction of total in vitro translation products of poly(A)⁺ RNA which was immuno-precipitated by anti-GAPDH antibody as described in Materials and Methods.

Figure 4: Dot-spot analysis of GAPDH and LDH-A mRNA content in poly(A)⁺ and poly(A)⁻ RNA from rat tissues.

50 μ g of total RNA from each tissue were loaded on a 100 μ l oligo(dT)-cellulose column (10) and the flow-through recycled three times. Poly(A)⁻ RNA was ethanol-precipitated from the final flow-through. After washing with 5 ml loading buffer, poly(A)⁺ RNA was eluted and precipitated. 1/20th of input total RNA, poly(A)⁺ and poly(A)⁻ RNA was dot-spotted in duplicate on two separate filters. They were then probed separately with inserts from pRGAPDH-1 and pRLD42 as described in Materials and Methods.

each tissue were run in parallel onto oligo(dT)-cellulose column under conditions where poly(A)⁺ globin mRNA was shown to be quantitatively retained and then eluted. After washing and elution, aliquotes of poly(A)⁺ and poly(A)⁻ fractions were dot-spotted in parallel with the equivalent amount of total RNA they were derived from and probed with pRGAPDH-1 insert. Scanning of the hybridization signals of Figure 4 (left panel) allows us to accurately determine the proportion of poly(A)⁺ and poly(A)⁻ GAPDH mRNA in each tissue. From the results of these measurements, it emerges that two situations with respect to the polyadenylation state of GAPDH mRNA were encountered in the various tissues analysed. In a first class of tissues, which includes liver, testis, heart and muscle, no more than 10% of total GAPDH mRNA is in poly(A)⁻ form. In another class, to which belong kidney and brain, only about 70% of GAPDH mRNA is in the poly(A)⁺ form. To rule out the possibility that the differential polyadenylation of GAPDH mRNA merely reflects a general situation characteristic of the tissue rather than of a specific messenger, we examined the polyadenylation status of another mRNA present in all tissues analysed here. For this purpose, we chose lacticodehydrogenase A mRNA (LDH-A) for which a cDNA probe (pRLD42) was available (32). The proportion of LDH-A mRNA in poly(A)⁺ and poly(A)⁻ forms was assessed as above. Results indicated that, at variance with the GAPDH situation, LDH-A mRNA is quantitatively polyadenylated in all tissues

(Figure 4, right panel). We can therefore conclude that the differential polyadenylation status observed for GAPDH mRNA is not only tissue-specific but also messenger-specific.

We have also investigated the in vitro translatability of GAPDH poly(A)⁺ RNA from the different tissues in the reticulocyte lysate cell-free system in the presence of (³⁵S) methionine. Results of Table 2 (column C) show that the amount of immunoprecipitable newly synthesized GAPDH reflects the abundance of its mRNA. We made no attempt at quantitating GAPDH message in poly(A)⁻ RNA of brain and kidney because the presence of high amounts of ribosomal RNA precludes reliable measurements.

GAPDH gene is transcribed at similar rates in the different rat tissues

An evaluation of the transcriptional activity of GAPDH gene in each tissue was obtained by measuring the amount of nascent GAPDH transcripts. To this aim, isolated nuclei were treated with pancreatic RNase and incubated in vitro in the presence of α (³²P) UTP under conditions allowing for elongation of nascent chains as described by Schibler et al. (28). As pointed out by these authors, two requirements must be met if hybridization signals are to reflect the actual number of RNA molecules engaged in GAPDH gene transcription: i) Preexisting unlabeled RNA, which could compete with labeled transcripts during the hybridization assay, must be eliminated prior to incubation. RNase A treatment of crude nuclei was shown to be efficient at this task since only short radioactive RNA molecules were detected after a one minute incubation, the length of which being only slightly longer than the 60-70 nucleotides expected to be protected by RNA-polymerase (28); ii) Elongation rates must be comparable in the different tissues examined. Electrophoresis of radioactive transcripts (not shown) after different times of incubation confirmed that their elongation rates were actually quite comparable (about 15-17 nucleotides per minute). Both prerequisites being satisfied, we could proceed to determine the proportion of GAPDH-specific sequences among nascent labeled transcripts by hybridizing them to filter-bound dots of pRGAPDH-1 DNA in large excess. As internal references we added DNA sequences coding for another house-keeping functions: plasmid pRLD42 encoding LDH-A (32), and one tissue-specific function: plasmid pRSA8 coding for rat serum albumin (33).

Hybridization signals presented in Figure 5 show that the GAPDH gene is transcribed with comparable efficiency in all tissues analysed. It also shows that: i) serum albumin is transcribed exclusively in liver and only at a 3 to 4 fold lower rate than GAPDH (these values were obtained after correction for the lengths of the probes (220 bp for pRGAPDH-1 and 850 bp for pRSA8).

Figure 5: Dot-spot assay of nuclear transcripts of various genes in rat tissues. 10^6 cpm of (^{32}P) labeled nascent nuclear transcripts obtained from each tissue as described in Materials and Methods were hybridized to nitrocellulose stripes carrying 5 μg DNA dots from pRGAPDH-1 (GAPDH), pRLD42 (LDH-A), pRSA8 (RSA) and pBR322. Crosses on heart, brain and kidney stripes indicate that pRSA8 was not spotted.

Considering that serum albumin mRNA is a major mRNA species in liver, this difference in transcription rates is surprisingly small. This discrepancy can be explained by a difference either in transport rate to the cytoplasm or in stability. There has been suggestions that RSA mRNA is particularly stable in liver (34). ii) LDH-A gene is transcribed at a rate similar in all tissues but about 2-fold higher than the GAPDH gene (after correction for the 800bp length of the pRLD 42 insert). The assay of the GAPDH gene activity on isolated nuclei indicates that the regulation of its expression occurs at the post-transcriptional level.

DISCUSSION

In this work we have investigated the expression of the GAPDH gene in various rat tissues. Western blot analysis have shown that the enzyme is present at quite different levels in muscle, heart, testis, liver, brain and kidney. Particularly, muscle and heart, which have the most intense glycolytic activity, also exhibited the highest GAPDH levels as has already been observed in human and rat (1). Except for testis which has not been analysed, relative abundances in all other tissues are also in good agreement with this previous report (1).

At the RNA level, the following facts have emerged: i) total GAPDH RNA content vary considerably from one rat tissue to another. ii) GAPDH gene is transcribed at the same rate in all rat tissues tested. From the above two points, we can already conclude that the regulation of this gene occurs at the post-transcriptional level. Precedents to this situation have been published recently. Casimir and Groudine (35) have shown that the chicken thymidine kinase gene is transcribed at the same level in dividing and

non-dividing cells while important variations of mRNA were observed. Cleveland and Havercroft (36) have shown that tubulin mRNA contents could be reduced in colchicin-treated CHO cells while transcription remained unchanged. However, we cannot rule out a subtle transcriptional control involving tissue-specific differences of the mRNA itself (possibly originating from different promoters), as was demonstrated in the case of the alpha-amylase I gene in mouse liver and salivary gland (37). Experiments are now in progress in our lab to compare GAPDH mRNA from different tissues at the sequence level. (iii) GAPDH mRNA is nearly quantitatively polyadenylated in all tissues but brain and kidney where only 70% behave as poly(A)⁺. Conversely, LDH A mRNA is quantitatively polyadenylated in all tissues. This control thereby demonstrates that the differential polyadenylation state of GAPDH mRNA is not only tissue-specific but also messenger-specific. This in turn suggests that the polyadenylation state might, at least in brain and kidney, be a post-transcriptional level of regulation in the expression of GAPDH gene. In any case, our data are clearly insufficient grounds for further speculation about the relevance of GAPDH mRNA polyadenylation to its tissue-specific expression. iv) There seems to be a good correlation between the steady-state levels of GAPDH protein and of its mRNA in different rat tissues. This suggests that GAPDH expression is not differentially regulated from one tissue to another at the translational or post-translational level. However this point needs to be confirmed by the study of half-lives of both GAPDH messenger and protein and the kinetics of in vivo GAPDH RNA translation. As a matter of fact, differential turnovers of messenger RNA has already been reported as a level of regulation for different specific mRNAs in the same cell (38-40) or for the same mRNA in the same cell in different circumstances (41,42). Tissue-specific differential protein turnover has also been reported in the case of mouse lactate dehydrogenase (43).

In chicken, Milner et al. (44) have shown that the amount of GAPDH protein is correlated with the level of its available poly(A)⁺ mRNA. We have also started to investigate the content of GAPDH protein and various forms of mRNA in chicken tissues. Preliminary results of such a study again support a model of post-transcriptional regulation, although in this case there seem to be tissue-specific differences in the polyadenylation state of GAPDH mRNA (Blanchard et al., results to be published).

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