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Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family

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ABSTRACT: We have isolated and sequenced a full-length cDNA clone encoding rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, E.C.1.2.1.12). The entire mRNA is 1269 nucleotides long exclusive of poly(A) and contains respectively 71 and 196 bases of 5' and 3' non-coding regions. Primer extension as well as S_1 nuclease protection experiments clearly established that a single (or at least a highly prominent) GAPDH mRNA species is expressed in all rat tissues examined. This sequence allowed the determination of the hitherto unknown primary structure of rat GAPDH which is 333 aminoacids long. Comparison between GAPDH sequences from rat, man and chicken revealed a high degree of sequence conservation at both nucleotide and protein levels.

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in the control of glycolysis, and consists in the assembly of four identical subunits. It is encoded by a single functional gene in man, mouse and chicken genomes (1-6) while no such information is available yet in rat. In a previous report, we showed that the tissue-specific differences in GAPDH content are regulated in rat at the post-transcriptional level (7). Recently, our laboratory showed that an unusually high number of GAPDH-related sequences (exceeding 200 copies) were present in the rat genome (8), making the prospect of isolating the functional gene(s) quite remote. This situation made it critical to investigate the possibility that different GAPDH mRNA might be expressed in different tissues. To this aim, we have constructed and sequenced a full-length rat muscle GAPDH cDNA clone and used it to look for mRNA sequence heterogeneity in various tissues. Moreover, comparative analysis of different vertebrate GAPDH mRNAs revealed strikingly conserved structural features suggesting that they might be involved in some post-transcriptional steps of regulation.

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MATERIALS AND METHODS:

Animals

Organs were excised from sacrificed 39 days-old inbred Wistar rats.

RNA preparation and Northern blots

Total RNA was extracted from rat tissues using a modification of the guanidinium thiocyanate-lithium chloride procedure of Cathala et al. (9). Poly(A)$^+$ RNA was selected through two cycles of oligo(dT)-cellulose chromatography as described (10). RNA samples were electrophoresed on 1.2% denaturing agarose gels containing 1M formaldehyde and transferred onto nitrocellulose membranes according to Thomas (11).

Construction and identification of GAPDH cDNA clones from rat muscle.

Double-stranded cDNA was synthesized as described by Gubler and Hoffman (12). Ds-cDNA was then dC-tailed and size fractionated through a Sepharose 4B column, equilibrated with 10mM Tris-Hcl (pH 7.5), 1mM EDTA and 100mM NaCl. Every one-drop fraction was annealed to oligo-dG tailed pBR322 vector (New England Nuclear) in a 1:1 molar ratio. $CaCl_2$ treated (13) E.coli DP50 (dap^-,thy^-) cells were transformed with the annealed mixture. Clones were screened on GeneScreen membranes (New England Nuclear) as described by Hanahan and Meselson (14), using the purified insert of pRGAPDH1, a partial cDNA clone from rat C6 glioma cells previously obtained in our laboratory (7). Positive clones were prepared using the alkaline method of Birnboin and Doly (15).

Determination of nucleotide sequence

The purified insert was restricted with four-cutters enzymes such as AluI, MboI and HaeIII, followed by random subcloning in M13 mp10 and mp11 (16). Sequencing was performed according to Sanger and Coulson (17). Additional restriction sites revealed by sequencing were used to complete the determination. Alternatively, the purified insert was 3' end-labelled by α^{32}P-ddATP and terminal transferase (18), cleaved with HinfI and then separated and sequenced according to Maxam and Gilbert (19).

Radioactive labeling of probes and autoradiography

Radioactive labeling of plasmids or purified inserts was done with the Amersham nick-translation kit as specified by the supplier. Autoradiographs were done on Kodak XAR film with a Quanta III Dupont Cronex intensifying screen at -70°C.

Oligonucleotide synthesis

The 5' A-A-G-G-G-G-T-C-G-T-T-G-A-T-G-G-C-A 3' oligonucleotide was synthesized by the phosphotriester method (20) on a solid phase polystyrene

resin (Bachem, California) and purified by gel filtration through a G-50 column followed by HPLC on a Waters C18 column (21).

Oligonucleotide primed cDNA synthesis

200 nanograms of oligonucleotide were labelled by T4-polynucleotide kinase (PL.Biochemicals) and α^{32}P-ATP (Amersham, 5000Ci/mmol). One fourth of the reaction was annealed with 5-10 µg of poly(A)$^+$ RNA for one hour at 42°C in a 10 µl mixture containing 50mM Tris-HCl (pH 8.3), 80mM KCl and 10mM $MgCl_2$. 10 ul of a mixture containing 10mM DTT, 2mM each cold dXTP and 20 units of AMV reverse transcriptase (Genofit,Switzerland) was then added. After one hour incubation at 42°C, the reaction was stopped by adding 10µl of a formamide-dyes solution (99.9% deionized formamide,10mM EDTA, 0.01% each bromphenol blue and xylene cyanol dyes). The mixture was then boiled for 5 minutes and loaded onto a 6% polyacrylamide gradient buffered gel (22). Run-off products were located by autoradiography, eluted from the gel and sequenced according to Maxam and Gilbert (19).

S1-mapping experiments

Approximately 1 µg of recombinant M13 single-stranded DNA was annealed for 1 hr at 55°C to 2 picomoles of sequencing primer in 10 µl of a mixture containing 15 mM Tris-HCl (pH 8.5), 15mM $MgCl_2$, 20 µCi α^{32}P-dATP (Amersham). Reaction was started by addition of 3 µl of 0.5mM each dCTP, dGTP, dTTP and 1 unit of DNA-polymerase I (Klenow fragment) and incubated for 45 minutes at 30°C. 1.5 µl of 100mM Tris-HCl pH 7.5, 100mM DTT, 500mM KCl, 100mM $MgCl_2$, 3 µl sterile H_2O, 2 µl sequencing chase solution, 1 unit of Klenow fragment and 40 units of EcoR1 were added, and incubation was pursued for 30 minutes at 37°C. The ^{32}P-labeled single-stranded insert was separated on a 5% polyacrylamide denaturing gel, located by autoradiography, and electro-eluted from the gel into a dialysis bag in 0.5X Tris-Borate-EDTA for 2 hrs at 400 volts. Probe was recovered by ethanol precipitation, and samples (20,000 cpm) were mixed with 0.5-2 µg poly(A)$^+$ RNA in a 50 µl mixture (pH 6.4) containing 80% formamide, 40mM Pipes, 1mM EDTA and 0.5M NaCl. Samples were heated for 10 min at 85°C, then hybridization was performed for 3hrs at 57°C (i-e 5°C higher than the melting temperature of the probe). 450 µl S1-buffer (30mM Na Acetate, 50mM NaCl, 1mM $ZnSO_4$, 0.5% glycerol) were added to each sample and reaction was started by the addition of 200 units of S1 nuclease. After 1 hr at 37°C, samples were ethanol precipitated, dissolved in 10 µl formamide-dyes solution (see above) and loaded on a 5% sequencing gel.

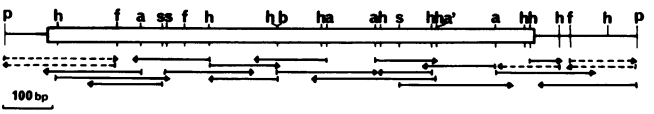


FIGURE 1: Partial restriction map of rat GAPDH cDNA and sequencing strategy. M13 shotgun subcloning was performed on various restriction digests of pRGAPD-13 insert. Arrows indicate the location and extent of each sequencing gel. Dashed arrows indicate regions sequenced according to Maxam and Gilbert. Abbreviations are: p: PstI, h: HaeIII, f: HinfI, a: AluI, s: Sau3A, b:BalI, a': ApaI.

RESULTS AND DISCUSSION

Characterization of a full-length GAPDH cDNA clone from rat muscle

3,000 ampicillin sensitive, tetracyclin-resistant colonies were screened using the α^{32}P nick-translated insert from pRGAPDH-1, a partial GAPDH cDNA clone previously obtained from C6 rat glioma cells (7). Screenings were performed under high stringency conditions of washings (0.1X SSC, 0.1% SDS, 60°C). Out of the 14 positive clones, three of them contained plasmids harbouring inserts of approximately 1,300 nucleotides long. Sequencing of one of them, called pRGAPDH-13, was performed using a combination of chemical and enzymatic methods according to the strategy outlined in Figure 1. Sequence was determined on both strands due to overlapping clones throughout the cDNA.

Nucleotide as well as inferred protein sequence are shown in Figure 2. Comparison with previously known protein (23) and nucleotide sequences (24, 25) unambiguously demonstrates that the sequence does encode the entire GAPDH. Alhough no poly(A) appears in this clone, sequence comparison with other clones from C6 glioma cells (unpublished results) in which addition of poly(A) occurred 15 nucleotides downstream the polyadenylation signal AAUAAA provided evidence that the 3' non-coding region of RGAPDH-13 was complete. Mechanisms that could have led to this loss of poly(A) were not further investigated. We then ensured that the insert was containing a full-length 5' non coding region by primer extension experiments. For this purpose, a synthetic primer whose 3' terminus was located 86 bases downstream the AUG start codon, was used to specifically initiate reverse transcription of the 5' terminal region of GAPDH mRNA. This primer was also used to initiate Sanger's dideoxy sequencing reactions on M13 templates carrying the relevant part of the insert.

Products obtained by both methods are shown in Figure 3 (panel A). At first sight, it appears that the GAPDH reverse transcript is shorter by two or

```
Tails  10                                                  50                                                  80
GGGGGGGGGAGG  CTCTCTGCTCCTCCCTGTTCTAGAGACAGCCGCATCTTCTTGTGCAGTGCCAGCCTCGTCTCATAGAC
                        98                                                  128
AAG ATG GTG AAG GTC GGT GTG AAC GGA TTT GGC CGT ATC GGA CGC CTG GTT ACC AGG GCT GCC
    M   V   K   V   G   V   N   G   F   G   R   I   G   R   L   V   T   R   A   A
                    158                                                 188
TTC TCT TGT GAC AAA GTG GAC ATT GTT GCC ATC AAC GAC CCC TTC ATT GAC CTC AAC TAC ATG
F   S   C   D   K   V   D   I   V   A   I   N   D   P   F   I   D   L   N   Y   M
            218                                                 248
GTC TAC ATG TTC CAG TAT GAC TCT ACC CAC GGC AAG TTC AAC GGC ACA GTC AAG GCT GAG AAT
V   Y   M   F   Q   Y   D   S   T   H   G   K   F   N   G   T   V   K   A   E   N
        278                                                 308
GGG AAG CTG GTC ATC AAC GGG AAA CCC ATC ACC ATC TTC CAG GAG CGA GAT CCC GTC AAG ATC
G   K   L   V   I   N   G   K   P   I   T   I   F   Q   E   R   D   P   V   K   I
    338                                                 368
AAA TGG GGT GAT GCT GGT GCT GAG TAT GTC GTG GAG TCT ACT GGC GTC TTC ACC ACC ATG GAG
K   W   G   D   A   G   A   E   Y   V   V   E   S   T   G   V   F   T   T   M   E
398                                                 428                                                 458
AAG GCT GGG GCT CAC CTG AAG GGT GGG GCC AAA AGG GTC ATC ATC TCC GCC CCT TCC GCT GAT
K   A   G   A   H   L   K   G   G   A   K   R   V   I   I   S   A   P   S   A   D
                        488                                                 518
GCC CCC ATG TTT GTG ATG GGT GTG AAC CAC GAG AAA TAT GAC AAC TCC CTC AAG ATT GTC AGC
A   P   M   F   V   M   G   V   N   H   E   K   Y   D   N   S   L   K   I   V   S
                    548                                                 578
AAT GCA TCC TGC ACC ACC AAC TGC TTA GCC CCC CTG GCC AAG GTC ATC CAT GAC AAC TTT GGC
N   A   S   C   T   T   N   C   L   A   P   L   A   K   V   I   H   D   N   F   G
                608                                                 638
ATC GTG GAA GGG CTC ATG ACC ACA GTC CAT GCC ATC ACT GCC ACT CAG AAG ACT GTG GAT GGC
I   V   E   G   L   M   T   T   V   H   A   I   T   A   T   Q   K   T   V   D   G
                668                                                 698
CCC TCT GGA AAG CTG TGG CGT GAT GGC CGT GGG GCA GCC CAG AAC ATC ATC CCT GCA TCC ACT
P   S   G   K   L   W   R   D   G   R   G   A   A   Q   N   I   I   P   A   S   T
            728                                                 758
GGT GCT GCC AAG GCT GTG GGC AAG GTC ATC CCA GAG CTG AAC GGG AAG CTC ACT GGC ATG GCC
G   A   A   K   A   V   G   K   V   I   P   E   L   N   G   K   L   T   G   M   A
        788                                                 818
TTC CGT GTT CCT ACC CCC AAT GTA TCC GTT GTG GAT CTG ACA TGC CGC CTG GAG AAA CCT GCC
F   R   V   P   T   P   N   V   S   V   V   D   L   T   C   R   L   E   K   P   A
            848                                                 878
AAG TAT GAT GAC ATC AAG AAG GTG GTG AAG CAG GCG GCC GAG GGC CCA CTA AAG GGC ATC CTG
K   Y   D   D   I   K   K   V   V   K   Q   A   A   E   G   P   L   K   G   I   L
        908                                                 938
GGC TAC ACT GAG GAC CAG GTT GTC TCC TGT GAC TTC AAC AGC AAC TCC CAT TCT TCC ACC TTT
G   Y   T   E   D   Q   V   V   S   C   D   F   N   S   N   S   H   S   S   T   F
    968                                                 998
GAT GCT GGG GCT GGC ATT GCT CTC AAT GAC AAC ATT GTG AAG CTC ATT TCC TGG TAT GAC AAT
D   A   G   A   G   I   A   L   N   D   N   I   V   K   L   I   S   W   Y   D   N
                                    1058                                                1088
GAA TAT GGC TAC AGC AAC AGG GTG GTG GAC CTC ATG GCC TAC ATG GCC TCC AAG GAG TAA GAA
E   Y   G   Y   S   N   R   V   V   D   L   M   A   Y   M   A   S   K   E   .
                        1118                                1148
ACCCTGGACCACCCAGCCCAGCAAGGATACTGAGAGCAAGAGAGAGGCCCTCAGTTGCTGAGGAGTCCCCATCCCAACTCAGC
   1278                                1208                                1238
CCCCAAACACTGAGCATCTCCCTCACAATTCCATCCCAGACCCCATAACAACAGGAGGGGCCTGGGGAGCCCTCCCTTCTCTCG
          1268                  Tails
AATACCATCAATAAAGTTCGCTGCACCCTC CCCCCCCCCCC
```

Figure 2: Nucleotide sequence and predicted aminoacid sequence of rat GAPDH.

Complete nucleotide sequence was worked out using the GEL program (26) of Intelligenetics (Palo Alto). The deduced aminoacid sequence is shown below the nucleotide sequence. The region to which the specific oligonucleotide hybridizes is underlined.

three nucleotides than its ds-cDNA counterpart (M13 analysis). This was eventually confirmed by sequencing the run-off product. We therefore conclude that the additional sequence, (G)12-A-G-G that appears upstream the

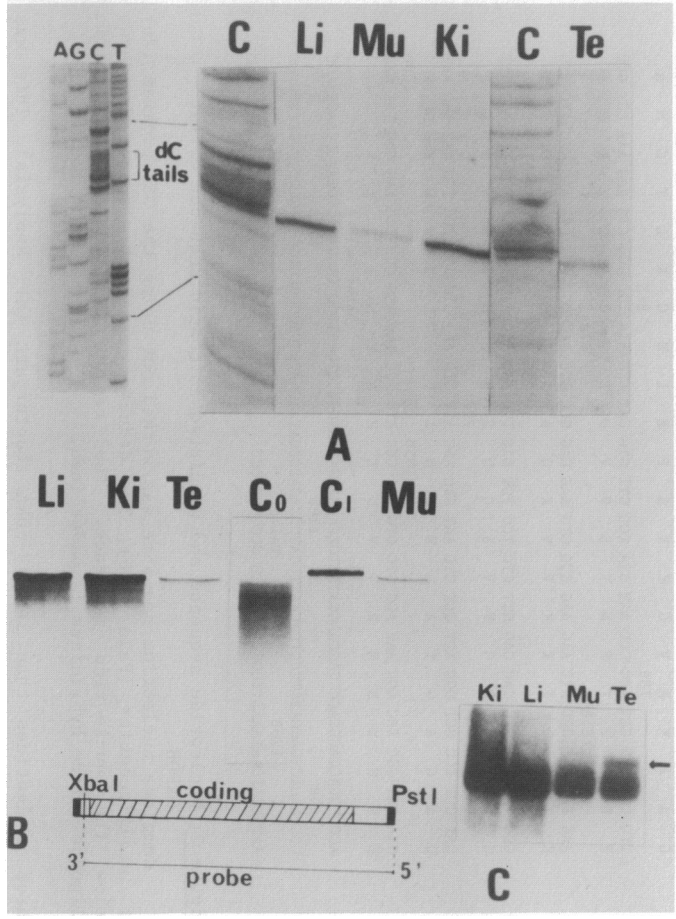


Figure 3: Analysis of rat GAPDH mRNAs in various tissues.

Panel A: Primer extension.
A regular dideoxy sequence of a 5' cDNA subclone initiated with the synthetic GAPDH primer is shown on the left. This same oligonucleotide was used to prime reverse transcription (see Methods). The 156 bp long run-off products obtained from kidney (Ki), liver (Li), testis (Te) and muscle (Mu) mRNAs, are shown on the right. Track C represents a dideoxyC sequencing gel of the same cDNA subclone as above except that the primer was 5' end labeled. Exposure time: 15 minutes.

Panel B: S_1-nuclease protection.
mRNAs from various tissues were hybridized to the uniformly ^{32}P-labelled probe (see below) and submitted to S_1-nuclease digestion. We include as controls yeast tRNA protection (C_o track) and undegraded probe (C_1 track). The slower migration in C_1 is due to salts present in S_1-nuclease reaction buffer. The lower diagram schematically represents the probe used for the protection. Filled boxes: dG/dC tails. Open boxes: non coding regions. Hatched boxes: coding region. Exposure time: 16 hrs except for track C_o (one week).

Panel C: Northern blot.
Hybridization was with nick-translated pRGAPD-13 insert. The arrow indicates the minor species observed in testis.

Equal amounts of poly(A)$^+$ mRNA were analysed in experiments A, B and C. Liver, kidney and testis: 5 µg, muscle: 0.5 µg.

mRNA sequence in the pRGAPDH-13 insert most likely represents the dG-tails, the dA residue resulting either from a point mutation that occurred during plasmid amplification, either from a cross-contamination of the nucleotide mixture used for the dC-tailing of ds-cDNA.

Identical major mRNA species is expressed in various rat tissues

GAPDH has been shown to be encoded by a unique coding sequence in all species so far analysed. However, to test the possibility that primary transcripts in various tissues might undergo differential splicing events, resulting in a unique coding region flanked by different tissue-specific non-coding regions, we analyzed both 5' and 3' non-coding regions of GAPDH mRNAs isolated from liver, kidney and testis. Figure 3 (panel A) shows the products of oligonucleotide primed cDNA synthesized on various messengers. Each of them yielded a major run-off product, the size of which being identical to that obtained from muscle mRNA. Chemical sequencing showed that their sequences were identical (data not shown). Thus, if more than one mRNA species is expressed to a significant extent, they have identical 5' non-coding regions and therefore must originate from the same promoter.

Primer extension being of no avail for investigating the 3' region of a mRNA, we had to resort to S1-nuclease protection experiment. Such an experiment is shown in Figure 3 (Panel B), using a single-stranded 1,258 bp long XbaI-PstI fragment (XbaI appears at position 31, Figure 2) as a probe. All four tissues display identical patterns: unique protected bands of identical length, the relative intensities of which are comparable to that obtained by primer extension experiments (Figure 3, Panel A). This latter point rules out the possibility that the primer used could have discriminated between different messengers. Taken together, these results suggest that the same predominant mRNA species is expressed in various tissues. However, should pRGAPDH-13 come from the shorter mRNA species, then no difference in size would show up when matched to larger mRNAs. Indeed, Northern blot analysis reveals a minor species of higher molecular weight in testis (Panel C) mRNA, which is not detected by S1-protection. We are currently investigating the exact nature of this minor band.

In conclusion, our results clearly demonstrate that a unique and identical major mRNA species is expressed in rat liver, muscle, kidney and testis, but leave open the possibility for larger minor mRNA species to be expressed. This might be the case in testis if the minor band observed in Northern blot turned out to be authentic GAPDH mRNA. Furthermore, as our study only deals with adult tissues, the possibility remains that different

Amino acids	1-90	91-260	261-333
Rat/Man	6.7	2.3	12.5
Rat/Pig	14.4	1.2	12.5
Rat/Chick	14.4	1.2	13.8
Man/Pig	11.1	3.5	6.9
Man/Chick	13.3	3.5	12.5

Table 1: Distribution of nucleotide substitutions within codons.

For each pairwise comparison and each region of the protein, aminoacid differences were expressed in percent. Man protein sequence is from Hanauer and Mandel (25).

mRNA species might be expressed during ontogeny, as recently suggested for human foetal and adult liver (27).

Evolutionary analysis of GAPDH sequences

i) Coding sequences: GAPDH protein is highly conserved through evolution.

Previous works from other laboratories have already reported a strong degree of conservation at the protein level (27, 28). Indeed, the present work reveals an internal string spanning from aminoacids 91 to 260 which is amazingly conserved in rat, man, pig (23) and chicken (Table 1). This region overlaps both domains of the protein (NAD binding and catalytic domains) and exhibits U.E.P.(Unit Evolutionary Period i-e the average time, in million of years, required for a 1% difference in amino acid sequence to arise between two lineages) ranging from 24 to 100, a value quite similar to that computed for histones (29).

Pairwise comparisons between rat, man (25) and chicken (24, 28) nucleotide sequences show that the vast majority of changes are transitions that affect the third base of codons (Table 2). As codon usage for GAPDH is similar in these organisms (data not shown), this would tend to suggest that non-conservative mutations have been strongly counterselected and even more so in the central part of the molecule. In any case, this eliminates the possibility that GAPDH conservation might be due to random fluctuations in the mutational pressure.

ii) Unexpected conservation of rat and man non-coding regions

While chicken non-coding regions differ from others to such an extent that they could not be properly aligned, we found extensive homologies

	1st	2nd	3rd
Rat/Man	17 (5)	8 (3)	80 (60)
Rat/Chick	24 (10)	11 (5)	115 (52)
Man/Chick	25 (7)	11 (4)	125 (60)

Table 2: Distribution of nucleotide substitution within codons. Rat, man and chicken coding sequences were aligned on the basis of their protein sequence. The total number of changes of each base of the triplet was scored for each pairwise comparison. The number of transitions is indicated in parentheses.

```
a)
Rat CTCTCTGCTCCTCCCTGTTCTAGAGACAGCCGCATCTTCTTGTGTGCAGT*GCCAGCC*******TCGTCT
Man ------------*------G-C--T--------------T-----*--C-------GAGCCACA---*--

    CATAGACAAG ATG
    -**------- ATG
b)
Rat GAAAACCCTGGACCACCCAG*CCCAGCAAGGATACTGAGAGCAAGAGAGAGGCCCTCAGTTGCTGAGGAGTC
Man --**C-----------*---C----------GC--*A----G---------A------C*-----G------

    CCCATCCCA*ACTCAGCCCCC**AACACTGAGCATCTCCC****TCACAATT*CCATCCCAGACCCCATAAC
    --**-G---C------T----CAC-------**-------CTCC-----G--T----G*T-------T-G-A

    AACAGGAGGGGCCTGGGGAGCCC*TCCCTTCTCTCGAATACCATCAATAAAGTTCGC**TGCACCCTC
    G-GG----------A--------CCA----**G--AT---------------A-C-TG---T-AGC-
```

Figure 4: Comparison of rat and man non coding sequences.
Sequences were aligned to maximum homology using Intelligenetics SEQ program (26). a:5' non coding region.b: 3' non coding regions.Man sequence is from Hanauer and Mandel (25) and from our laboratory (unpublished results). Stars indicate deletions.

between those of rat and man (Figure 4). The human GAPDH pG1 sequence published by Arcari et al. (27) was excluded from the comparison because this clone appears to be rearranged at its extremities. The 5' non-coding region lacks the last 19 nucleotides and contains a 40 bp long inversion. The 3' non-coding region differs by several point mutations and additions from four independent and identical human GAPDH sequences: two retrogenes on the X chromosome (25, 30) and cDNA sequences from human fetal liver and Hela cells (unpublished results of this laboratory). However, homologies between rat and man are unusual in some respects:

- Since the divergence of rat and man occurred, non-coding regions have accumulated mutations only twice faster than the coding region (Figure 4).

- The extent of homology (80% excluding deletions) is similar to the one observed (82 %) between the corresponding non-coding regions of mouse β minor and β major globins, which arose since rodents have diverged.

- GAPDH belongs to a multigenic family (8, 25), in which non-coding regions exhibit the same level of reiteration than coding sequences (Piechaczyk et al., results to be published). If no selective pressure is exerted on the primary structure of non-coding regions, as can be inferred from comparative analysis of various eucaryotic messengers (31), we would expect them to accumulate mutations at an even faster rate because of gene correction mechanisms.

- We found in both rat and human 5' non-coding sequences an

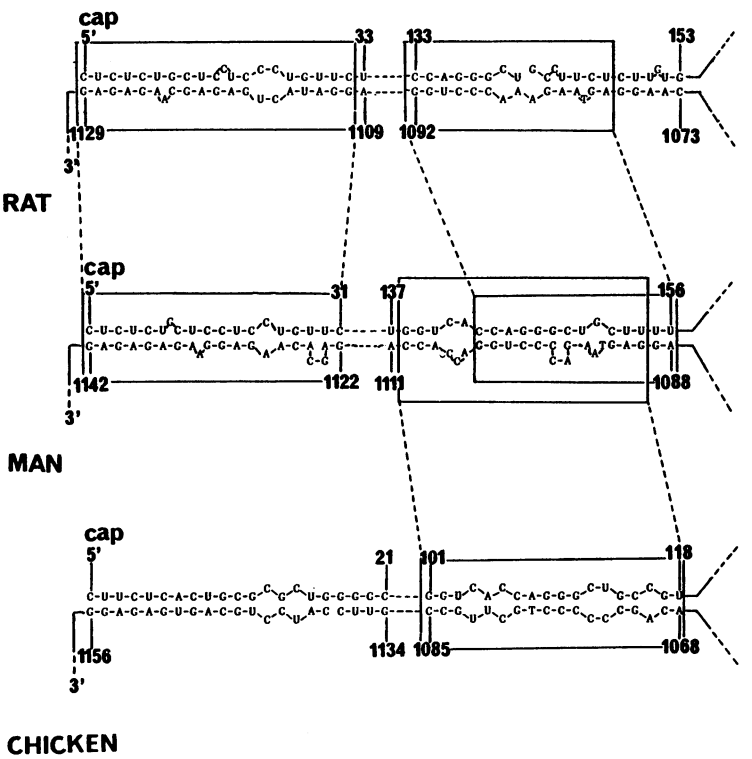


Figure 5: Conserved structural features of various vertebrate GAPDH mRNAs.

Numbers refer to published sequences: Rat (Figure 2, this paper), Man (25), and chicken (24). Boxes joined with dashed lines indicate mRNA regions of high homology. Only those base-pairings that have the lowest value of free energy were selected. Computation was made using Intelligenetics SEQ program (26).

11-nucleotides box which shows a strong complementarity to the 3' end of 18S ribosomal RNA:

		37	47
Rat	5'	UUCUUGUGCAG	3'
18S rRNA	3'	AGGAAGGCGUC	5'
Man	5'	UUCUUUUGCGU	3'
		36	46

Although this feature has previously been shown for other messengers (32), no such degree of base pairing was observed.

- Computer analysis revealed that putative base pairings involving 5' and 3'non-coding regions appear conserved in rat, human and even chicken

sequences (Figure 5).

For all these reasons, we suggest that GAPDH non-coding regions might carry some important signals, possibly for translational regulation or for mRNA stability, which might explain differential tissue-specific expression (7).

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