Age-Related Changes in Glucose Transporter-One mRNA Structure and Function (44185)

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Abstract. **To determine the molecular mechanisms of age-related changes in the expression of glucose transporter 1 (GLUT-1) mRNA in cerebral tissue, male Fischer 344 rats at 4, 12, and 24 months of age were studied. The GLUT-1 mRNA in cerebral tissue was not significantly different among the various age groups. The *in vitro* translatability of GLUT-1 mRNA of 24-month-old rats (0.867 ± 0.066 arbitrary units) was significantly lower than that in 4-month-old (1.403 ± 0.153) $P < 0.01$ or 12-month-old rats (1.387 ± 0.122) $P < 0.01$. The poly (A) tail length of GLUT-1 mRNA decreased from 200–350 nt in 4-month-old rats to only 50–100 nt in 24-month-old rats. Twelve-month-old rats also showed reduced poly (A) tail lengths. The poly (A) tail of G_3PDH mRNA was not altered with age. The changes in GLUT-1 mRNA translatability did not correlate with GLUT-1 content in total cerebral tissue homogenate or in isolated cerebral microvessels, suggesting that GLUT-1 protein turnover is altered with age. It is concluded that aging is associated with significant changes in the structure and function of GLUT-1 mRNA.** [P.S.E.B.M. 1997, Vol 216]

Aging is associated with a host of changes in the blood-brain barrier (BBB) (1, 2). Of particular interest is the age-related reduction in the BBB transport of glucose (3, 4). Glucose is the principal substrate of the central nervous system (CNS), and almost all the glucose that enters the CNS is transported across the BBB by glucose transporter 1 (GLUT-1). In the cerebral tissue, the most abundant GLUT-1 isoform is 45 kDa. This is believed to be the neuronal and glial GLUT-1. The BBB specific GLUT-1 isoform is 55 kDa. Less than 5% of GLUT-1 identified in total cerebral homogenates is the 55-kDa isoform which is localized at the BBB (5). The deglycosylated isoform of GLUT-1 in cerebral tissue is 38 kDa. The functional role of this latter isoform is not known.

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Although under normal conditions the transport of glucose is not rate limiting for cerebral metabolism, under certain conditions such as during hypoglycemia, the BBB glucose transport becomes a rate-limiting step in cerebral glucose utilization (6). The precise molecular mechanisms underlying the age-related changes in GLUT-1 are not clear.

Previously published work on age-related changes in GLUT-1 has shown that although immunoreactive mass of GLUT-1 does not change with age the cytochalasin B–binding sites, another measure of glucose transporter, are reduced (4). To explain this apparent discrepancy, we hypothesized that an age-related reduction in GLUT-1 clearance may increase the chances of post-translational changes in the tertiary structure of GLUT-1, thereby reducing its binding sites without altering its mass. Because of inherent difficulties in the *in vivo* turnover studies the *in vitro* GLUT-1 synthesis was measured and was found to be reduced in aged rats. Since the length of poly (A) tail is a determinant of mRNA translation, this parameter was also determined in rats at various ages.

Materials and Methods

Experimental Animals. Male Fischer 344 rats at 4, 12, and 24 months of age were obtained from the National Institute of Aging Colony maintained by Harlan Industries (Indianapolis, IN). The food intake of the rats stabilized

within 3 days of their arrival to our animal facilities. The rats were maintained on regular rat chow and tap water *ad libitum*. Within 2 weeks of arrival, the rats were sacrificed by exsanguination from the abdominal aorta under pentobarbital anesthesia (45 mg/kg intraperitoneally). The internal organs and blood smear were inspected and serum creatinine was measured. Rats with internal organ malignancy or renal failure were discarded from the study.

Isolation of Cerebral Microvessels. The forebrain was removed, stripped of dura, and processed as described previously (4). Briefly, the rat forebrains were placed on a chilled surface and the superficial blood vessels removed. Forebrains from each group were pooled and placed in a preweighed beaker of chilled buffer containing Krebs-Ringer HEPES, pH 7.4, with 0.5% bovine serum albumin (BSA). The tissue was weighed and homogenized with seven strokes in a glass homogenizer especially milled to give a clearance of 0.25 mm. An equal volume of 26% dextran in buffer was added and the sample was centrifuged at 1000*g* for 10 min. The supernatant containing the bulk of the cellular debris and lipid was suctioned off and the adjacent sides of the centrifuge tubes were wiped dry. The pellet contained both large and small vessels as well as cellular contaminants. The pellets were resuspended in chilled buffer and poured through 80-μm nylon mesh. The microvessels passed through the mesh and were then poured through a column containing 0.45- to 0.50-mm glass beads retained with 45-μm nylon mesh. The microvessels, retained on the glass beads, were eluted off by gently swirling the glass beads with buffer and collected by centrifugation (1000*g* for 10 min). The microvessels were washed twice in Krebs-Ringer and resuspended in 0.05 *M* Tris-HCl buffer, pH 7.4, and stored frozen at −70°C. Purity of the preparations was determined by light microscopic examination of microvessels stained with 0.1% toluidine blue. Phase-contrast microscopy was also done for some preparations. The activity of the enzyme γ-glutamyltransferase (GGT), a marker for brain capillary endothelium (7) was measured according to the procedure of Orlowski and Meister (8). Alkaline phosphatase activity was measured with a Sigma diagnostic kit (Sigma Chemical Co., St. Louis, MO) using the method of Bessey *et al.* (9) with some modifications. Protein measurements were done using the method of Lowry *et al.* (10). The details of the biochemical characteristics and purity of isolated microvessels from rats at different ages have been published previously (4).

Western Blot Analysis of GLUT-1 Mass. Cerebral microvessel proteins (10 μg) or proteins from total cerebral homogenates (10 μg) of rats at different ages were electrophoresed in denaturing (SDS), 10% polyacrylamide gel under reducing conditions (11). Proteins in the gels were electrophoretically transferred to nitrocellulose paper (12). GLUT-1 on nitrocellulose paper was probed with rabbit anti-rat GLUT-1 antibody (East Acres Biologicals, Southbridge, MA) at a final dilution of 1:5,000 for 2 hr at room temperature. Horseradish peroxidase–linked goat anti-rabbit IgG was used at a final dilution of 1:10,000 for 1 hr at room temperature. Blots were developed using enhanced chemiluminescence (ECL) Western blotting technique (ECL Kit) as described by the manufacturer (Amersham, Inc., North Chicago, IL). The synthetic peptide consisting of the 13 amino acids of the carboxy terminal of rat GLUT-1 was also purchased from East Acres Biologicals. The antiserum preadsorbed with this synthetic peptide was used as internal negative control. The same blots were stripped and reprobed with anti-alkaline phosphatase antiserum (Accurate Chemical and Scientific Corp., Westbury, NY) at 1:5,000 dilution and processed as described above. The stripping of the blots was carried out by treating nitrocellulose membrane with 0.2 *M* glycine at room temperature for 30 min. Subsequently, the membrane was washed twice with phosphate-buffered saline (pH 7.4) at room temperature treated with ECL and exposed to x-ray film overnight. The film was then developed to ascertain complete removal of the probe.

The densitometry was carried out using the personal densitometer from Molecular Dynamics (Sunnyvale, CA). The bands were analyzed after subtraction for background and GLUT-1 band intensity was normalized against the alkaline phosphatase bands. The alkaline phosphatase activity in cerebral microvessels isolated from rats at different ages was not different.

Northern Blot Analysis of GLUT-1 mRNA. Cerebral RNA was isolated by cesium chloride density gradient centrifugation (13). The GLUT-1 cDNA was prepared as follows. RNA (10 μg) from rat fetal brain at Day 15 was reverse transcribed with MMCV-reverse transcriptase, then directly amplified with 30 cycles using a 5′ primer corresponding to the sequence of GLUT-1 cDNA at 1464 to 1583 and a 3′ primer corresponding to GLUT-1 sequence of 1756 to 1775 (14). The 312-bp fragment was gel purified and subcloned into the *SmaI*/*Hinc*II site of PGem 3Z and sequenced with Sequenase Kit purchased from United States Biochemicals Co. (Cleveland, OH). There were only two substitutions; an A for C at 1602 and A for G at 1710 site. This 312-bp GLUT-1 cDNA fragment was used for Northern hybridization. RNA (20 μg) from the brains of the rats in various groups were electrophoresed in 1.5% agarose gels in the presence of 2.2 *M* formaldehyde (15), transferred to the nylon membrane by diffusion blotting, and finally hybridized with the GLUT-1 cDNA fragment (16). The membrane was dried and exposed to x-ray film for autoradiography. The same blots were stripped and reprobed with G_3PDH cDNA (obtained from American Tissue Culture Collection, Rockville, MD) as internal control. The stripping was carried out by placing the membrane in a box containing 0.1 × SSC preheated at 100°C and the box was kept in boiling water for 20 min. The membrane was rinsed with 0.1 × SSC at room temperature and exposed to x-ray film overnight. The film was then developed to ascertain complete removal of the probe.

The end labeling of the cDNA was carried out with terminal transferase using previously published method

(17). The GLUT-1 and G_3PDH mRNA bands were quantitated with the personal densitometer purchased from Molecular Dynamics.

***In Vitro* Translational Efficiency of GLUT-1 mRNA.** Poly (A) RNA was prepared by oligo (dT) cellulose chromatography as described previously (18). The *in vitro* translation procedure employed rabbit reticulocyte lysate and [^{35}S]methionine and cysteine (>800 Ci/mmol; New England Nuclear, Boston, MA) (19). One microgram of poly (A) RNA from the brains of rats at different ages was translated in the presence of 25 μCi (2 μl/reaction tube) of [^{35}S]methionine and cysteine using an *in vitro* translation kit (Life Technologies Inc., Grand Island, NY), according to the manufacturer's protocol. The extent of incorporation of the radiolabel was determined by TCA precipitation. GLUT-1 in the *in vitro* translation mixture was immunoprecipitated using rabbit anti-rat GLUT-1 IgG and *Staphylococcus aureus* suspension (20). Immunoprecipitated GLUT-1 was resolved by 10% reducing SDS-PAGE. The gel was enhanced, dried, and exposed to x-ray film for autoradiography to quantitate the 38-kDa GLUT-1 translated *in vitro*.

Evaluation of the Status of Poly (A) Tail of GLUT-1 mRNA Transcripts. Total RNA from rat cerebra was digested with RNase-free DNase 1 for 30 min at 37°C, phenol extracted, ethanol precipitated, and dissolved in water. RNA preparations were reverse transcribed using oligo (dT) containing a 5′ adaptor sequence 5′-GCGAGCTCCGCGGCCGCG(T)n (21). The cDNAs were amplified with adaptor/dT radiolabeled with polynucleotide kinase and γ [^{32}P]ATP (22) as the 3′ primer and 5′-ATCTTGGAGCTGTTCCGCTC sequence obtained from the 3′-coding region of the GLUT-1 cDNA as the 5′ primer. This pair of primers is referred to as Pair A in Figure 1. The amplification procedure was 1 min at 93°C, 1 min at 60°C, and 2 min at 72°C for 35 cycles, plus an additional 5 min at 72°C for 1 cycle. In mock reactions, reverse transcriptase was omitted. The resulting products were passed through the quick-spin column (Qiagen) to remove unincorporated adaptor/dT primer. These products were digested with *Stu* I, electrophoresed on 8% nondenaturing polyacrylamide gel and autoradiographs were obtained. A 1-μl aliquot of the amplified products were reamplified using the same 5′ primer and a 3′ primer (5′-CGGAAGCGATCTCATCGAAGG) complimentary to the 20 nucleotides of the coding region close to the 3′ untranslated region of GLUT-1 cDNA. This is referred to as Pair B in Figure 1. These products were digested with *Nco* I, and the digested and undigested products were resolved on a 2% agarose gel and stained with ethidium bromide.

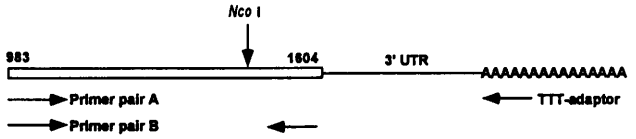

Figure 1. Schematic diagram of the strategy for the poly (A) tail analysis. cDNAs were prepared from animals at different age groups with the adaptor/dT primer and amplified with the indicated primer pairs. Nucleotide 983 and 1604 indicate the initiation of 5′ primer and 3′ primer, respectively. The 3′ untranslated region (3′ UTR) is shown with a thin bar. The position of the *Nco* I site within the translated region of mRNA is indicated. Primer pair A consists of oligo dT/adapter sequence as the 3′ primer and a 20 mer (nt 983–1002) as 5′ primer. The primer pair B consists of the same 5′ primer and a 20 mer (nt 1583–1604) as the 3′ primer. The diagram is not to scale.

To establish the specificity of the findings, the poly (A) tail length of G_3PDH mRNA was measured with the same technique. For these studies, the 5′ primer used was 19-mer oligomer extending over 972 to 990 nucleotides of the 3′-coding region of the G_3PDH mRNA (5′-TGAATATGGCTACAGCAAC-3′).

Statistical Analysis of Data. Results are presented as mean ± SE. Analysis of variance with Bonferroni's correction was used to assess the significance of differences between means. A P value <0.05 was considered significant.

Results

GLUT-1 Protein Content. The 55 kDa isoform of GLUT-1 in cerebral microvessels from 4-month-old rats in arbitrary units was 49.6 ± 7.2, in 12-month-old rats it was 62.0 ± 6.0, and in 24-month-old rats was 56.1 ± 9.6. The differences did not reach statistical significance. No other GLUT-1 isoforms could be found on these gels. In each age group, five independent preparations of cerebral microvessels were examined. Each microvessel preparation was obtained from seven to eight rat cerebra.

The immunoblots of proteins from total cerebral homogenates (n = 10 in each age group) indicated a lack of age-related difference in 45-kDa isoform of GLUT-1. Thus, this isoform in 4-month-old rats (18559.6 ± 1617.0) was not significantly different from 12-month-old (19688.8 ± 848.7) or 24-month-old rats (17160 ± 1266.4). In these blots the 55-kDa isoform was less than 5% of total GLUT-1.

GLUT-1 mRNA Content of Cerebral Tissue. Northern blots of cerebral RNA from rats revealed a single species of mRNA at 2.6 kb. Ten rats in each age group were studied. The intensity of GLUT-1 mRNA band was normalized against control hybridization with G_3PDH cDNA. There were no age-related differences in GLUT-1 mRNA content of rat cerebral tissue.

Determination of the *in Vitro* Translation Efficiency of GLUT-1 mRNA. The SDS-PAGE electrophoresis of *in vitro*–translated products which were immunoprecipitated with anti GLUT-1 antiserum is shown in Figure 2. A 38-kDa band representing deglycosylated GLUT-1 was found (Fig. 2A). The GLUT-1 counts were normalized against the radioactivity incorporated in the total translation products. The *in vitro* synthesis of GLUT-1 from mRNA of 24-month-old rats (0.867 ± 0.066 arbitrary units) was significantly lower compared with 4-month-old controls (1.403 ± 0.153), or 12-month-old rats (1.38 ± 0.122) $P < 0.01$.

Estimation of Poly (A) Tail Length of GLUT-1 mRNA. Total RNA from cerebral tissue of rats at different

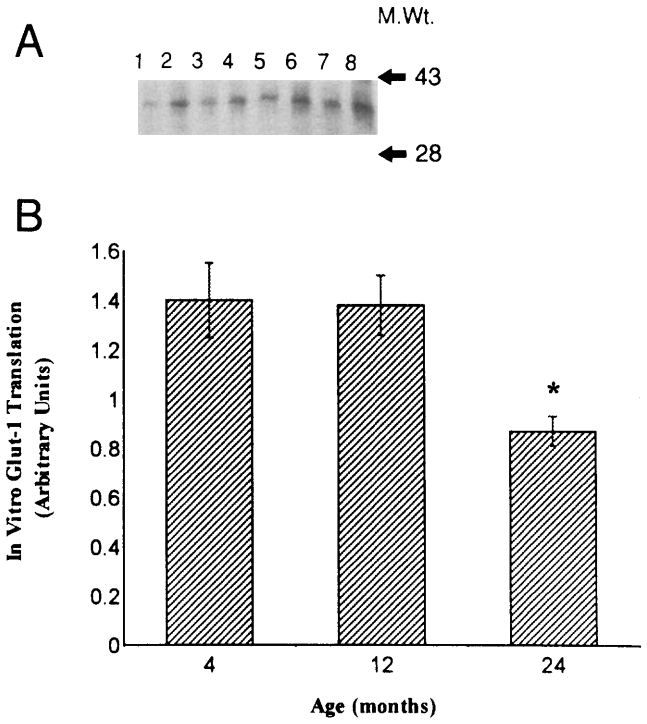

Figure 2. Messenger RNA (1 μg) from 24- (Lanes 1–3), 12- (Lanes 4 and 5) and 4- (Lanes 6–8) month-old rats was *in vitro* translated in a rabbit reticulocyte lysate system. GLUT-1 in *in vitro*–translated mixture was immunoprecipitated with anti-rat GLUT-1 antiserum, and analyzed by SDS-PAGE and autoradiography. A 38-kDa band is visible. Mean ± SEM of optical density (O.D.) of *in vitro* synthesized GLUT-1 mass. $^*P < 0.05$ compared with 4-month-old rats; $n = 6$ in each age group.

ages was reverse transcribed using oligo (dT) containing a 5′ adaptor sequence 5′-GCGAGCTCCGCGGCCGCGTTT. The priming for reverse transcriptase can occur anywhere along the stretch of (A) residues in the poly (A) tail, 3′ untranslated region, or 3′-coding region generating cDNA products of variable length. If the mRNA has extensive poly (A) tail, the cDNA products will be heterogenous, with some products that are larger than the largest cDNA product from a mRNA with a shorter poly (A) tail. If the poly (A) tail is short, a more homogeneous class of shorter products should be produced. The cDNAs were amplified with adaptor/dT radiolabeled with γ [^{32}P]ATP as the 3′ primer (the adaptor serves to anchor the primer and prevents progressive shortening of the products during amplification) and a GLUT-1–specific 5′-ATCTTGGAGCTGTTCCGCTC sequence obtained from the 3′-coding region of the GLUT-1 cDNA as the 5′ primer. These products were digested with *Stu* I, and analyzed by autoradiography.

The total length of GLUT-1 cDNA to the end of 3′ untranslated region in nucleotides (nt) is 2571. The 5′ primer, a 20 mer, ranges from nt 983 to 1002. The PCR products were digested with *Stu* I (has only one site in the cDNA from 5′ primer to the end), which cuts at position 2163 in AGGCCT sequence between G and C. Any PCR products less than 408 nt in length were attributed to cDNAs primed by oligo (dT) in the 3′ untranslated and 3′-coding regions. Any PCR products equal to or bigger than 408 nucleotides are due to cDNAs generated by priming in the poly (A) stretch.

The products of PCR were analyzed by an 8% nondenaturing polyacrylamide gel, followed by autoradiography (Fig. 3). In older rats, the largest product is approximately 500 nt long (Fig. 3, Lanes 3 and 4), whereas in young rats (4 months old) (Fig. 3, Lane 2) the largest product is approximately 750 nt long. Assuming that the longest product in each case was due to priming at the extreme 3′ end of the poly (A) tail, and since the translated and untranslated regions of GLUT-1 mRNA beyond *Stu* 1 cut site is 408 nt, then one can estimate the maximum length of poly (A) tail in young animals as approximately 350 nt whereas in aged animals it is approximately 100 nt. Since molecular weight standards on a polyacrylamide gel separate on the basis of size as well as charge, the sizes of PCR products are not absolutely accurate. Keeping all these considerations in mind, the size of poly (A) tail determined on the basis of these data is approximately 200–350 nt or larger in the 4-month-old animals and 50–100 nt in 12- and 24-month-old animals.

To ascertain the specificity of the technique and to verify that we are indeed working with the GLUT-1 mRNA region, ranging from 5′ primer to the end of the poly (A) tail, the original PCR products were reamplified by the same 5′ primer and a common 3′ primer derived from the extreme 3′ end of the GLUT-1 cDNA (Primer Pair B, see methods of procedures). As expected, a PCR product of 621 bp was amplified (Fig. 4, Lanes 5–7). These reamplified products did not include a poly (A) stretch of the 3′ untranslated region. When the products of this reamplification were digested with *Nco* I, two bands, a 384-bp and a 237-bp, were generated in each case (Fig. 4, Lanes 2–4). Thus, the differences observed in the PCR products (408 nt or larger in size) generated by using oligo/dT as a PCR primer were due to differences in the poly (A) tail length. Comparison of the length of the poly (A) tail of G_3PDH mRNA transcripts from young and aged rats is shown in Figure 5. There were

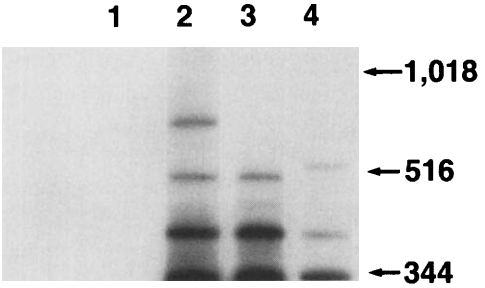

Figure 3. Comparison of the length of poly (A) tail of GLUT-1 mRNA transcripts from 4-, 12-, and 24-month-old rat brains. cDNAs were prepared using primer pair A shown in (Fig. 1). PCR amplified products were electrophoresed in 8% polyacrylamide gel and visualized by autoradiography. A high–molecular weight band seen in 4-month-old rat brain transcript (Lane 2) is absent from 12- (Lane 3) or 24- (Lane 4) month-old rat brain transcripts. Lane 1 is the mRNA in a mock reaction in which reverse transcriptase was omitted.

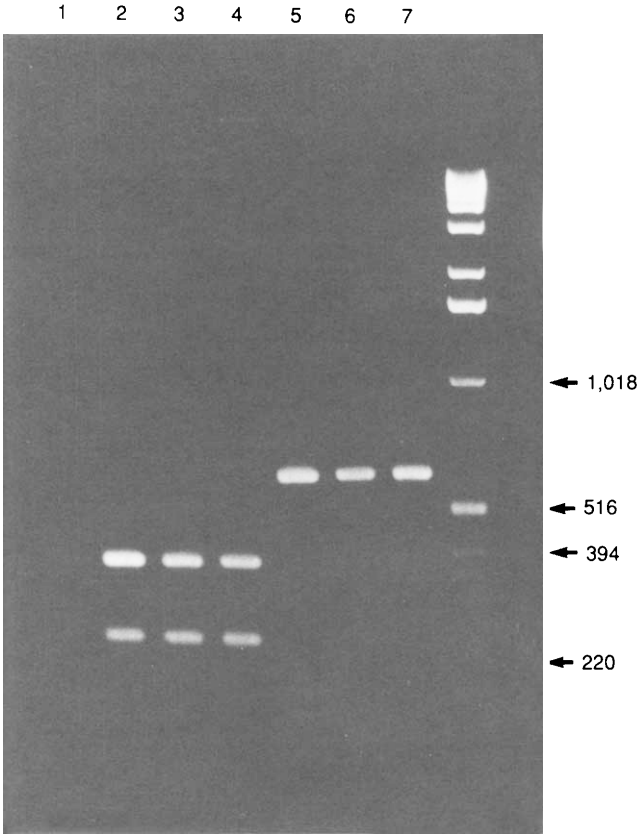

Figure 4. Reamplification and restriction enzyme digestion of PCR products from Figure 3. PCR products (1 μl each) from Lanes 1, 2, and 3 from Figure 3 were reamplified using Primer Pair B (Fig. 1). These products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. The expected 621-bp fragment was amplified in each sample from 4-, 12-, and 24-month-old rats (Lanes 5, 6, and 7, respectively). These reamplified PCR products were digested with *Nco* I, electrophoresed in a 2% agarose gel, and stained with ethidium bromide (Lanes 2–4). Two bands, a 384-bp fragment and a 237-bp 3′-terminal fragment were obtained as expected. Lanes 2, 3, and 4 correspond to the products shown in Lanes 5, 6, and 7, respectively. Lane 1 corresponds to Lane 1 in Figure 3, where there are no products since reverse transcriptase was omitted in the mock reaction.

no differences in the size of various products identified even when the films were overexposed.

Discussion

There were no age-related changes in GLUT-1 mRNA or GLUT-1 protein content of cerebral tissue. Thus, the age-related reduction in the BBB transport of glucose is probably not due to a reduction in the GLUT-1 transporter of cerebral microvessels. Therefore, it would appear that the membrane environment of the transporter changes with age, or the distribution of GLUT-1 between the membrane and microsomal pools may be altered or perhaps the turnover decreases and some post-translational modification of GLUT-1 occurs, reducing maximum rate of transport. The direct measurements of GLUT-1 turnover with age require many aged rats, making the cost of the experiment prohibitive. Alternatively, one can measure the synthetic rate of GLUT-1, which, in the light of unaltered steady state content of cerebral GLUT-1, would serve as an indirect indicator of GLUT-1 turnover. Many proteins in aging animals have reduced turnover rate (23). In this study, the *in vitro* translational efficiency of GLUT-1 mRNA corrected for total mRNA translation was found to be significantly reduced in 24-month-old rats. This observation, taken together with unaltered GLUT-1 content, indicates that GLUT-1 turnover is also reduced in aged rats. The reduced *in vitro* translational efficiency may be the result of changes in mRNA structure, particularly its poly (A) tail length. Thus, the structure of poly (A) tail of GLUT-1 mRNA was studied.

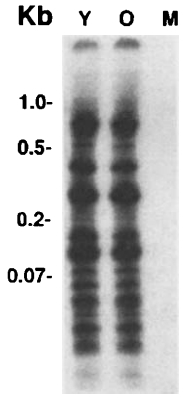

Figure 5. Comparison of the length of poly (A) tail of G_3PDH mRNA from 4- (Y) and 24- (O) month-old rat brains. cDNAs were prepared with the adaptor/dt primer and a 19 mer (nt 972–990) as 5′ primer from the coding region of G_3PDH mRNA. PCR amplified products were electrophoresed in 8% polyacrylamide gel and visualized by autoradiography. Lane M is mRNA from young rat that was reverse transcribed without reverse transcriptase in a mock reaction. There were no differences in the size of the products from various animal groups even when the film was overexposed for 24 hr.

The reduction in poly (A) tail of GLUT-1 mRNA was evident as early as 12 months of age and persisted in the 24-month-old age group. A similar change in hepatic albumin mRNA with age was recently reported (24). The change in poly (A) tail length does not occur in all mRNA species since the poly (A) length of G_3PDH mRNA of cerebral tissue was not reduced in aged rats (Fig. 5). Thus, it appears that shortening of poly (A) tail of some mRNAs is another mechanism of modulation of gene expression in aging animals. It is noteworthy that a similar reduction in poly (A) tail length of GLUT-1 mRNA was seen in diabetic rats (17). The age-related changes we hereby report simulate those found prematurely in diabetic rats. This is consistent with the view that diabetes, at least in certain tissues, is a model of premature aging (25). The possibility that the age-related changes could have been secondary to glucose intolerance of aged rats was excluded by the finding of normal serum fructosamine concentrations in 24-month-old rats (131 ± 5.4 μ*M*) compared with 4-month-old rats (147 ± 7.3 μ*M*).

The biological significance of the reduced poly (A) tail length of GLUT-1 mRNA with age is not clear. Although poly (A) tail length of mRNAs has been shown to be an important modulator of mRNA stability and translatability

(26–28), our observations in 12-month-old rats indicate that other factors in addition to poly (A) length must be involved in determining the age-related changes in GLUT-1 mRNA translatability. One such factor is the involvement of the 5′-cap binding proteins (26–28). Future studies should focus on the interaction of poly (A) tail and 5′-cap in modulating mRNA functional changes with age.

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