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Partial purification and characterization of the mRNA for human thymidine kinase and hypoxanthine/guanine phosphoribosyltransferase

(microinjection/autoradiography/methylmercuric hydroxide/agarose gel/urea/polyacrylamide gel/HeLa cell)

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ABSTRACT **We used direct microinjection of poly(A)$^+$RNA into individual hypoxanthine/guanine phosphoribosyltransferase-deficient or thymidine kinase-deficient cells and detected the specific *in vivo* translation products as an assay for human hypoxanthine/guanine phosphoribosyltransferase or thymidine kinase mRNAs. The incorporation of [^{3}H]hypoxanthine or [^{3}H]thymidine into cells in response to injected mRNA was assayed *in situ* by autoradiography. Methylmercuric hydroxide/agarose gel analysis showed that human hypoxanthine/guanine phosphoribosyltransferase mRNA contains ≈1,530 nucleotides, which is twice the number required for its protein coding capacity. The mRNA for human cytoplasmic thymidine kinase is estimated to be approximately the same length; thus, the size of the cytosol thymidine kinase subunit can be predicted to be ≈47,000 daltons, if the full coding capacity of its mRNA is utilized.**

The genes for hypoxanthine/guanine phosphoribosyltransferase (HPRT; EC 2.4.2.8) and thymidine kinase (TK; EC 2.7.1.21) are perhaps the most widely studied loci in mammalian cell genetics. HPRT, a purine salvage enzyme, catalyzes the transfer of phosphoribose from 5-phosphoribosyl 1-pyrophosphate to hypoxanthine or guanine bases to yield IMP or GMP and pyrophosphate. The native human HPRT is a multimeric enzyme composed of identical subunits whose molecular size has been determined by $NaDodSO_4$/polyacrylamide gel electrophoresis to be 24,000–26,000 daltons (1, 2). There is no evidence that the subunit alone possesses catalytic activity (3). Human and mouse HPRTs are dimeric, but the dimers can readily associate to form tetramers (4). HPRT deficiency is the fundamental biochemical defect in humans suffering from the Lesch–Nyhan syndrome, and its partial deficiency causes gout and arthritis (5). Human genetic studies have indicated that the structural gene for HPRT is on the X chromosome (6, 7).

TK, an enzyme of the pyrimidine salvage pathway, catalyzes the phosphorylation of thymidine to dTMP (8, 9). The metabolic significance of TK is unclear. This enzyme is involved not only in the utilization of exogenous thymidine but also in the activation of thymidine analogs or deoxyuridine analogs that are currently used in cancer or viral chemotherapy (10). It is generally agreed that the activity of TK increases in cells during DNA synthesis (11, 12). HeLa cells contain two molecular forms of mitochondrial TK and one cytosol (soluble) enzyme. They are different in sedimentation coefficient and other properties (13). Furthermore, Adler and McAuslan (14) have shown that the expression of different forms of TK is a function of the replicative state of cells. Human cytoplasmic and mitochondrial TK genes map to chromosomes 17 and 16, respectively (15, 16).

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A number of attempts have been made to purify TK from various mammalian sources, but it is difficult to draw general conclusions about the molecular weight and the kinetic properties of the enzyme. Kit *et al.* (17) and Lee and Cheng (18) have reported that the molecular weight of human cytoplasmic TK is 90,000 and that of the mitochondrial form is 70,000. However, Ellimo and Van der Weyden (19) demonstrated by $NaDodSO_4$/polyacrylamide gel electrophoresis that the subunit molecular weight of TK from human adult liver, presumably the mitochondrial form, is 48,000. They also have reported that the smallest native molecular form has a molecular weight, as determined by gel filtration, of 49,000; the enzyme was usually found as multimers with molecular weights of 98,000 and 340,000.

Stacey and Allfrey (20) used microinjection to transfer duck globin mRNA into HeLa cells and observed efficient translation. Graessmann and Graessmann have studied the function of early simian virus 40-specific mRNA by microinjection into mouse kidney cells (21). Liu *et al.* (22) have utilized similar techniques to introduce human mRNA specifying a number of cellular proteins into specific mutant cells. The biological activity of a specific mRNA was detected by the complementation of the mutant phenotype by *in vivo* translation of the injected mRNA. This microinjection method for assaying mRNA is simple and provides greatly enhanced sensitivity in detecting translation products from small amounts of mRNA. In this report, using microinjection to detect human HPRT and TK mRNA together with denaturing gel fractionation methods, we have partially purified these two mRNAs and determined their size. The size of human HPRT mRNA is estimated to be ≈1,530 bases, which is 2 times longer than its required coding sequence. The molecular size of HeLa TK mRNA, presumably coding for cytoplasmic enzyme, is also about 1,530 bases; if most of these are used in translation, the size of the cytosol TK subunit is predicted to be about 47,000 daltons.

MATERIALS AND METHODS

Cell Cultures. HeLa cells were grown in RPMI 1640 medium (GIBCO) containing 5% fetal bovine serum, penicillin, streptomycin, and kanamycin in suspension culture. HT1080 HPRT$^-$ (a human rectal sarcoma line deficient in HPRT) and LTK$^-$ (a mouse L cell deficient in TK and adenine phosphoribosyltransferase activity) were grown in Dulbecco–Vogt modified Eagle's medium (GIBCO) with 10% fetal bovine serum.

Abbreviations: HPRT, hypoxanthine/guanine phosphoribosyltransferase; TK, thymidine kinase.

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Preparation of Cytoplasmic Poly(A)$^+$RNA. HeLa cell cultures containing 5×10^9 cells were pelleted and washed twice with saline. Subsequently, the cells were dispersed and resuspended in 50 ml of ice-cold buffer containing 30 mM Tris·HCl (pH 7.4), 30 mM KCl, 0.1 M NaCl, heparin (1 mg/ml), and 0.15% Nonidet P-40. When the cell membranes were lysed completely, nuclei and mitochondria were removed by centrifugation at 9,500 rpm for 10 min with a Sorvall SS34 rotor. The supernatant was made 10 mM in EDTA and immediately mixed with 0.5 vol of buffered phenol. After the addition of NaDodSO$_4$ to 0.5% and 0.5 vol (of the supernatant) of chloroform/isoamyl alcohol, 24:1 (vol/vol), the supernatant was thoroughly extracted. The aqueous phase was repeatedly extracted with an equal volume of phenol/chloroform/isoamyl alcohol, 24:24:1 (vol/vol), until no interface material was apparent and then was extracted twice with chloroform to remove residual phenol. Total cytoplasmic RNA then was precipitated with ethanol. Poly(A)$^+$RNA was purified twice by oligo(dT)-cellulose (Collaborative Research) column chromatography, precipitated with ethanol, and dissolved in 1 mM Tris (pH 7.5) for microinjection at 1 mg/ml or in water for gel electrophoresis.

Fractionation by Urea/Polyacrylamide Gradient Gel. Electrophoresis in urea gels was performed by a modified procedure of Spradling *et al.* (23) and Gross *et al.* (24). Poly(A)$^+$RNA (in 0.05 M NaOAc, pH 5.6/3.5 M urea/10% sucrose/0.05% bromophenol blue/0.05% xylenecyanol FF) was heated at 65°C for 3 min, quickly chilled, and fractionated on a 3.25–7% linear polyacrylamide slab gel. The running buffer was 90 mM Tris·HCl, pH 8.3/90 mM boric acid/4 mM EDTA/0.1% NaDodSO$_4$ (buffer 1); 3.25% polyacrylamide (29:1 ratio of acrylamide to bisacrylamide) was made in buffer 1 containing 7 M urea, whereas 7% polyacrylamide was made in the same buffer but with 8 M urea. The stacking gel consisted of 2.8% polyacrylamide/7 M urea in buffer 1 (pH 6.7). This was used to prevent the disruption of the gradient during the insertion of a slot former. The dimensions of the slab gel were 15 × 23 × 0.18 cm. Gels were polymerized overnight prior to use. Electrophoresis was for 21 hr at 210 V. A mixture of HeLa 28S and 18S rRNA and *Escherichia coli* 23S and 16S rRNA (Miles) supplied molecular weight standards for RNA and was electrophoresed in parallel with the sample. These unlabeled RNA bands were localized by 254-nm UV absorption with Dupont Cronex Lightning Plus XL fluorescence screen underneath the gel. Care was taken not to irradiate the HeLa mRNA in the adjacent sample lane. The fractionated RNA was cut from the gel (4 mm per fraction) and extracted with 50 mM Tris·HCl, pH 7.4/0.1 M NaCl/5 mM EDTA/0.1% NaDodSO$_4$ overnight at room temperature. The RNA was further purified by centrifugation to remove gel debris, followed by oligo(dT)-cellulose column chromatography. Eluted RNA was then recovered by precipitation with ethanol in the presence of carrier human tRNA and dissolved in 1 mM Tris·HCl (pH 7.4).

Fractionation by Methylmercuric Hydroxide/Agarose Gel. The procedures used have been described (25, 26). Briefly, HeLa cytoplasmic poly(A)$^+$RNA (100 μg) was electrophoresed through a 1% agarose/10 mM CH$_3$HgOH (Alfa-Ventron, Beverly, MA) slab gel (13 × 13 × 0.3 cm) in borate buffer (0.1 M boric acid/6 mM sodium borate/1 mM EDTA/10 mM sodium sulfate, pH 8.2) at room temperature. Electrophoresis was for 1 hr at 40 V, followed by 18 hr at 25 V. Unlabeled marker RNA (200 μg of HeLa 28S and 18S rRNA and 200 μg of *E. coli* 23S and 16S rRNA) were electrophoresed in adjacent lanes. Their mobilities were identified by UV absorption as described above. The gel was then sliced manually into 2-mm fractions. Gel fractions were mixed with 0.2 ml of buffer (10 mM Tris·HCl, pH 7.4/0.1 M NaCl/10 mM EDTA/0.2% NaDodSO$_4$/1 mM 2-mercaptoethanol), melted at 95°C for 5 min, and frozen at −70°C. After thawing and removing the collapsed agarose by centrifugation, we extracted the supernatant once with phenol/CHCl$_3$/isoamyl alcohol, 24:24:1 (vol/vol), and once with chloroform/isoamyl alcohol, 24:1 (vol/vol). RNA was precipitated with alcohol in the presence of carrier yeast tRNA. Finally, RNA pellets were washed with 2 M LiCl and then with ethanol and dissolved in 30 μl of 1 mM Tris (pH 7.5).

Microinjection Procedure. Microinjection of RNA solutions into the cytoplasm of individual cells was performed by the method of Graessmann (27). The recipient cells were grown on coverslips (25 mm × 25 mm) containing photoengraved microscopic grid patterns (28), which facilitate the localization of injected cells after autoradiography. The injection pipettes were prepared from glass capillaries with fine glass filaments attached to their inner walls (1.2 mm o.d., American Glass, Bargaintown, NJ) to facilitate sample loading. The vertical pipette puller (model 700C) was from David Kopf Instruments, Tujunga, CA. Injection was carried out on an inverted phase-contrast microscope (Leitz Diavert, ×200). Movement of the micropipettes was controlled with Leitz micromanipulators. As recipients, HT1080 HPRT$^-$ cells were injected individually. Since LTK$^-$ cells are small, they were fused with polyethylene glycol (1,540 or 3,000) for 30–45 sec to form polykaryons containing three or four cells to facilitate the injection process. RNA samples of 5–10 μl were centrifuged at 60,000 × *g* for 45 min in glass capillaries to remove debris that might clog the injection pipette. The glass capillaries were spun in a Beckman ultracentrifuge (SW 40 rotor) with homemade adaptors to hold the capillaries. After centrifugation, samples were withdrawn with glass needles and loaded into injection micropipettes. The volume injected into each single cell was $\approx 5 \times 10^{-11}$ ml (average value).

Assay for Enzyme Activity by Autoradiography. After injection with polyadenylated RNA, HT1080 HPRT$^-$ cells were incubated in Dulbecco–Vogt modified Eagle's medium supplemented with 10% fetal bovine serum containing [^{3}H]hypoxanthine (3.8 Ci/mmol; 1 mCi/ml) at 5 μCi/ml (1 Ci = 3.7×10^{10} becquerels). Injected LTK$^-$ cells were incubated in the same medium with [^{3}H]thymidine (20 Ci/mmol; 1 mCi/ml) at 5 μCi/ml. Incubation was stopped after 16 hr; cells were washed with saline, fixed with methanol/acetic acid, 3:1 (vol/vol), washed with cold 5% trichloroacetic acid and then with ethanol, and air dried. The coverslips were mounted on glass slides, dipped in Kodak NTB-2 emulsion, and exposed for 2 days.

RESULTS

Assay for Human HPRT and TK mRNA by Microinjection. Figures 1 and 2 depict the results of an autoradiographic assay for HPRT and TK mRNA activity. In order to assay for HPRT mRNA, human HT1080 HPRT$^-$ cells were injected with cytoplasmic poly(A)$^+$RNA from HeLa cells at 1 mg/ml (Fig. 1). After incubation with [^{3}H]hypoxanthine for 16 hr, the cells were washed, fixed, and autoradiographed. The injected cells incorporated label into DNA and RNA because of the *in vivo* translation of injected HPRT mRNA. Uninjected cells were always negative. The majority of injected cells incorporated radioactivity into RNA, and hence, only the cytoplasms were heavily labeled. In Fig. 2, polyethylene glycol-fused murine LTK$^-$ cells were used as recipients for the injection of HeLa cytoplasmic mRNA. Expression of TK mRNA was scored by the incorporation of [^{3}H]thymidine into nuclear DNA. We noticed that in polykaryons which received HeLa mRNA, every cell expressed TK activity. This is probably due to the fact that cells in S phase induce DNA synthesis in other mitotic compartments after cell fusion (29).

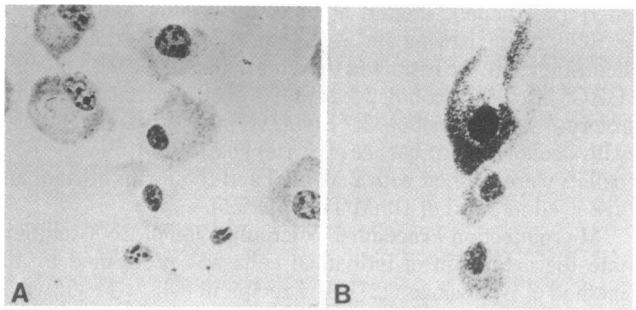

FIG. 1. Autoradiographic analysis for HPRT activity present in HT1080 (HPRT$^-$) cells before (*A*) and after (*B*) direct cytoplasmic injection with poly(A)$^+$RNA from HeLa cells. After injection, cells were fed with medium containing [^{3}H]hypoxanthine (5 μCi/ml) for 16 hr, followed by autoradiography.

These results indicated that microinjection and complementation of mutant phenotypes provide sensitive bioassays for HPRT and TK mRNA, and that this system can be extended as a general method for mRNA activity assays.

Analysis of Human HPRT mRNA by Methylmercuric Hydroxide Gel. Methylmercuric hydroxide is known to fully denature RNAs, even those with high guanosine-plus-cytosine content, and reliable molecular size measurements of RNA molecules as high as 4.0 megadaltons can be made on 0.5–1.0% agarose after reaction with 10 mM methylmercuric hydroxide (30). Furthermore, biologically active RNA can be recovered after gel fractionation (26)—an essential factor for our RNA assay. To determine the molecular size of human HPRT mRNA, we have electrophoresed HeLa cytoplasmic poly(A)$^+$RNA through 10 mM CH_3HgOH/1% agarose gels. The localization of HPRT mRNA on gels was evidenced by the expression of HPRT enzyme activity (incorporation of [^{3}H]hypoxanthine into cells) after the injection of the fractionated RNAs into human HT1080 HPRT$^-$ cells. In typical experiments, 150–200 cells were injected for each fraction. The RNA fractions ranging from the sample origin to the ≈40,000-daltons position were checked for HPRT activity. In the positive fractions, greater than 80% of the injected cells showed enzymatic activity. In two experiments, mRNA coding for HPRT activity was obtained in a single peak near 16 S (Fig. 3). The position of the peak is equivalent to 1,530 nucleotides in length (346 daltons per nucleotide) (Fig. 4). Calibration of molecular sizes was accomplished by plotting the square root of the marker RNA standards against logarithmic relative mobilities (30) with a least-squares method.

Gel Electrophoresis Analysis of Human TK mRNA. The size of HeLa TK mRNA was determined by two methods. HeLa cytoplasmic poly(A)$^+$RNA was first fractionated on a 3.25% polyacrylamide/7 M urea–7% polyacrylamide/8 M urea gradient gel. TK mRNA was identified by elution of RNA fractions from gel slices and then tested for activity by injection into murine LTK$^-$ cells. Approximately 150 recipient cells (polykaryons) were injected for each fraction. The expression of injected TK mRNA was monitored by the incorporation of [^{3}H]-thymidine into nuclei. TK activity was tested in fractions between 150,000 and 600,000 daltons. In two experiments, RNA in the 16-S region showed TK activity.

In a second procedure, we electrophoresed HeLa cytoplasmic mRNA through a 10 mM CH_3HgOH/1% agarose gel under fully denaturing conditions. RNAs from the gel origin to the 40,000-dalton position were checked for TK activity by microin-

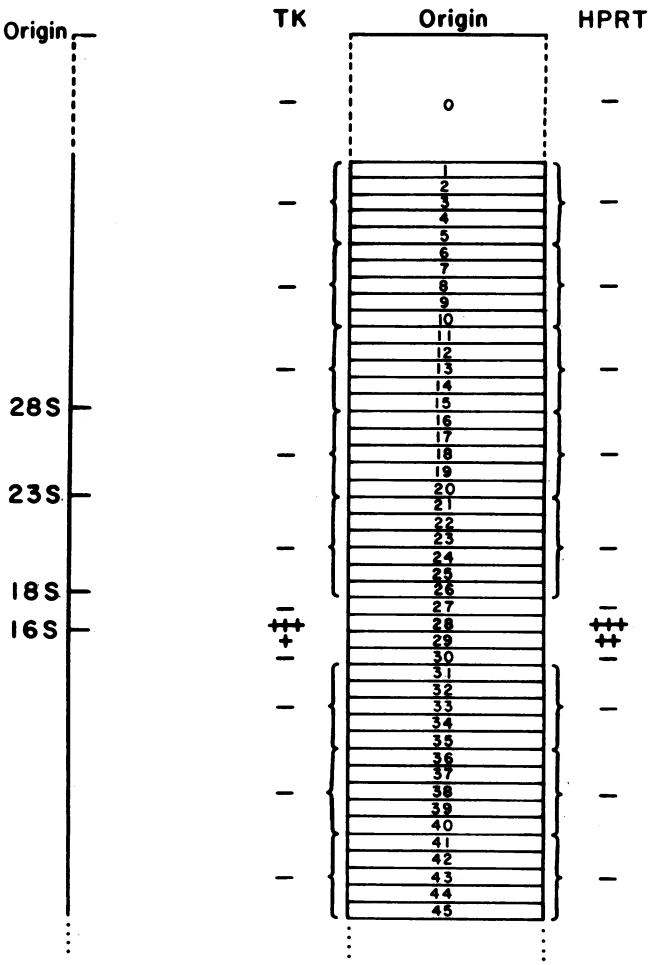

FIG. 3. Methylmercuric hydroxide/agarose gel fractionation of HeLa poly(A)$^+$RNA for sizing the HPRT and TK mRNAs. HeLa cytoplasmic poly(A)$^+$RNA was electrophoresed on a 10 mM CH_3HgOH/1% agarose slab gel. The gel was sliced into 45 fractions (2 mm per fraction); fractions 6–45 contained mRNA ranging from 4 megadaltons to 40,000 daltons. The position of HPRT and TK mRNA were recognized by injection of RNA eluted from gel slices into HPRT$^-$ or TK$^-$ cells and expression of enzyme activities. RNA from groups of five fractions were combined and injected as an initial screen, except for fraction 0. Fraction 0, an unsliced 18-mm gel near the origin, was injected as a single sample. Individual samples were then injected in the positive regions to further refine the position of the strongest signal. Signals were judged by the relative number of silver grain counts in autoradiographs: negative (−), weakly positive (+), moderately positive (++), and strongly positive (+++). Ribosomal RNAs from human (28 S, 18 S) and *E. coli* (23 S, 16 S) were used as size markers and run adjacent to the sample. Marker RNAs were identified by UV absorption bands.

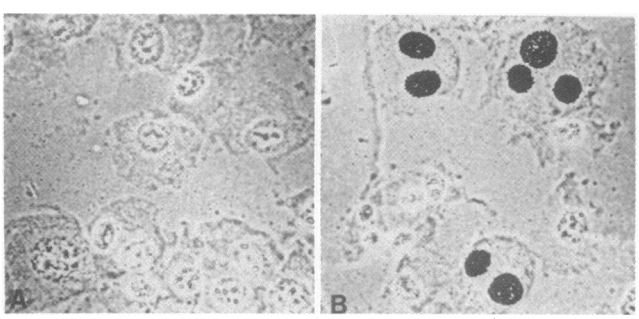

FIG. 2. Autoradiographic analysis for TK activity present in uninjected LTK$^-$ cells (*A*) and cells injected with poly(A)$^+$RNA from HeLa cells (*B*). The polykaryons were injected at 3 or 4 hr after fusion with polyethylene glycol. After injection the cells were incubated with [^{3}H]thymidine (5 μCi/ml) for 16 hr and subsequently autoradiographed.

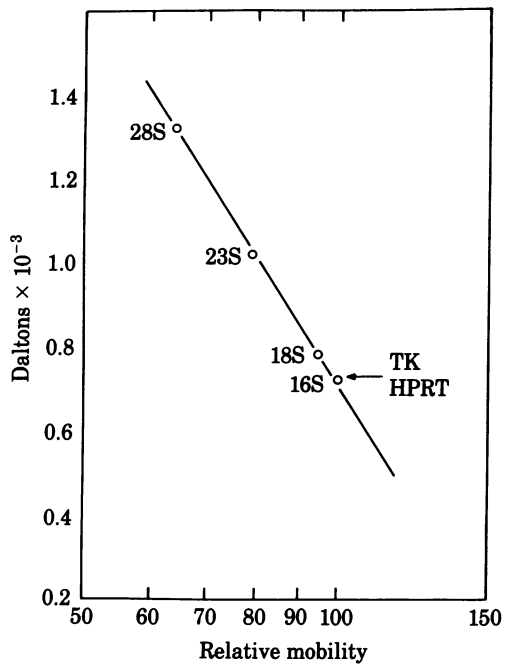

FIG. 4. Relationship of marker RNA molecular sizes and migration distances with interpolated values for HPRT and TK mRNA. The relative migration of all RNA species and the positions of HPRT and TK mRNA were determined as described in Fig. 3. Results were the average of two gels. The molecular sizes for marker RNA species were taken to be 1.75, 1.05, 0.605, and 0.525 megadaltons, respectively, for 28S, 23S, 18S, and 16S RNA species (31). The calibration line was plotted as described by Lehrach *et al.* (30) by a least squares method.

jection. In two experiments, TK mRNA was found in a single peak; the electrophoretic mobility of the peak was slightly slower than the 16S marker RNA (Fig. 3). This result indicated that human TK mRNA is ≈1,530 nucleotides in length (see Fig. 4).

DISCUSSION

We have successfully used a somatic cell microinjection method to assay for human HPRT and TK mRNA. This method has a number of advantages over traditional *in vitro* translation. It is simple, rapid, and sensitive. Moreover, if deficiency mutants are used as recipients for injection, one can study the relevant mRNA without detailed knowledge of the gene product, providing a phenotypic assay is available. The method can be applied to study any specific mRNA sequence for which a biological, immunological, autoradiographic, or selective assay is available. Using this RNA bioassay to monitor the purification of human HPRT and TK messages on methylmercuric hydroxide/agarose or urea/polyacrylamide gel, we have characterized their molecular size. Because it was difficult to quantitate the amount of mRNA injected and the level of enzyme expressed in recipient cells, we were not able to estimate absolutely the purity of our messages. However, based on the distribution of RNA on the separation gels, we estimate a minimal enrichment of the two messengers of 50–100 fold.

The subunit of the human HPRT enzyme has been determined by $NaDodSO_4$/polyacrylamide gel electrophoresis and is reported to be 24,000–26,000 daltons (1, 2). From this, the minimum HPRT coding sequence can be calculated to be about 700 nucleotides. If we assume that eukaryotic mRNAs contain up to a 200-base-long poly(A)$^+$ sequence (32), our estimate of 1,530 bases for the size of human HPRT mRNA suggests that it contains a considerable number of extra nucleotides not required to code for the polypeptide. During the course of this work, Brennand *et al.* (33) have reported that the predominant murine HPRT mRNA species is about 1,600 nucleotides long by using a cloned HPRT cDNA as a probe. They also detected an additional RNA species around 6,500 nucleotides long.

The size of the active human cytoplasmic thymidine kinase was determined by glycerol gradient and gel filtration to be 90,000 daltons and that of mitochondrial thymidine kinase was 70,000 (17, 18). However, Ellims and Van der Weyden (19) report that the smallest native molecular form of human mitochondrial TK has a molecular weight of 49,000 as determined by gel filtration, and the enzyme consistently associated to form dimers and hexamers. Here we report the partial purification of HeLa TK mRNA, presumed to be the cytoplasmic form, because it has been demonstrated that 97% of HeLa TK activity is cytoplasmic (34). We estimate the size of HeLa TK mRNA to be about 1,530 nucleotides long. Thus, it is not long enough to code for a 90,000-dalton protein. If one assumes 300 nucleotides for poly(A) plus untranslated leader sequences, we calculate that the TK protein has ≈410 amino acids [(1530 − 300)/3] and a molecular size of 47,000 daltons. This result suggests that the active human cytosolic enzyme is a dimer, taking into account the reports of Kit *et al.* (17) and Lee and Cheng *et al.* (18).

It is interesting to note that the cytoplasmic mRNA that codes for the herpes simplex virus type 1-specific TK sediments at 14.5 S. This corresponds to an RNA of 1,300 nucleotides, excluding the poly(A) tail (35, 36). The herpes simplex virus TK polypeptide has been determined to be ≈42,000 daltons; the size of the active HSV TK enzyme is ≈70,000 daltons (37, 38, 39). The TK gene of herpes simplex virus is ≈1,300 nucleotide pairs and is not interrupted by intervening DNA sequences (36, 40, 41, 42). The size of chicken TK genes is ≈1,000–1,400 base pairs (43). Therefore, there is a general similarity between the molecular sizes of human, herpes, and avian TK genes.

The mRNAs of human HPRT and cytoplasmic TK are similar in size. TK mRNA exhibits the same mobility on both urea/polyacrylamide and methylmercuric hydroxide gels as expected. However, in experiments not reported here with urea/polyacrylamide gels, we have obtained a higher molecular size value for the HPRT mRNA (≈2,600 nucleotides). We discount the validity of this result because urea gels are less denaturing than methylmercuric hydroxide gels. We assume that the different electrophoretic properties of HPRT and TK mRNA must be in some way attributable to differences in their base composition.

Our results have demonstrated that mRNA microinjection and complementation of mutant phenotypes provide a general method for mRNA assay. Combining mRNA bioassay and denaturing gel fractionation, we have characterized the sizes of HPRT and TK mRNAs. Our methods allow one to study the structure of eukaryotic mRNAs without being limited to systems that yield large amounts of RNAs, or a reliance on available cDNA probes. In the case of HPRT, our results provided information on transcriptional and translational processing. In the TK system, our results help to resolve the molecular size of the human cytosol TK subunit and provide information about the molecular structure of biologically active enzymes. Moreover, the size characterization and the partial purification of these two mRNAs should facilitate their molecular cloning.

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