Isolation and Cell-Free Translation of Messenger Ribonucleic Acids Specifying Thymus-Leukemia Antigens†

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ABSTRACT: Messenger ribonucleic acid (mRNA) for thymus-leukemia antigens, membrane-associated glycoproteins of murine leukemia cells, was obtained from polysomes of murine leukemia cells forming thymus-leukemia antigens. Polysomes forming thymus-leukemia antigens were recovered by immunoprecipitation using alloantibodies specific for the 1,2,3 determinants of the thymus-leukemia antigen complex before they were extracted with phenol–detergent. Poly(adenylic acid)-containing RNA [poly(A)-RNA] was fractionated by oligo[deoxythymidylate] [oligo(dT)]–cellulose chromatography. The mRNA obtained had a sedimentation coefficient of ~17 S in agreement with the predicted size necessary for forming proteins specifying thymus-leukemia antigens. In a wheat germ system, the polypeptides formed upon addition of mRNA for thymus-leukemia antigens consisted of a major product with a molecular weight of 42 000. It was larger than the nonglycosylated heavy chain molecule of 40 000 daltons formed by the cells themselves. On the cell surface thymus-leukemia antigens exist as glycosylated molecules of 47 000 daltons associated with a light-chain equivalent to β_2-microglobulin. Molecules of 40 000 daltons were isolated from the cells cultured in the presence of tunicamycin, an inhibitor of de novo glycosylation, and by treating thymus-leukemia heavy chains with endo-β-*N*-acetylglucosaminidase H.

Thymus-leukemia (TL)[1] antigens, glycoproteins associated with the membranes of thymus cells of some but not all mouse strains (Old et al., 1963; Old & Stockert, 1977), are detected by immunologic means on the surfaces of developing "immature" cells. Mature, thymus-derived cells in the spleen and other peripheral lymphoid organs capable of engaging in antibody "helper" functions and immune cytotoxic reactions spontaneously cease forming TL antigens; they are TL negative. Murine leukemias of thymus origin may express TL antigens reflecting their less differentiated, more embryonic state (Boyse et al., 1967, 1968a,b; Cohen & Liang, 1976; Liang & Cohen, 1977). Four TL antigenic determinants have been identified (Boyse et al., 1968a).

In the presence of specific antibodies, the quantity of TL antigens associated with the surface membranes of normal and neoplastic cells diminishes. The cells convert to TL antibody and complement resistance, a phenomenon termed antigenic modulation (Lamm et al., 1968). Antibody-induced reductions in the quantities of TL antigens are a consequence of antigen metabolism. The rate of degradation of TL antigens complexed with antibodies on the cells' surface is more rapid than the rate of antigen synthesis and replacement, leading to a gradual diminution in the number of antigens present (Yu & Cohen, 1974; Cohen & Liang, 1976). Antibody-induced "patching" and "capping" precede endocytosis of the complexed determinants.

A partial characterization of TL antigen structure has been performed. At the membrane, the molecule consists of two polypeptide chains—a smaller one of ~12 000 daltons equivalent to mouse β_2-microglobulin and a larger one carrying the determinants of the TL antigen complex (Yu & Cohen, 1974; Ostberg et al., 1975). The larger polypeptide is an integral membrane protein; its carboxy terminus is "buried" in the membrane. It is conceivable that TL antigens are present as dimers consisting of two identical TL-specifying proteins and two β_2-microglobulin molecules assembled into a four-chain polypeptide structure (Anundi et al., 1975).

"TL-like" determinants have been detected on the surface membranes of human thymus cells (Chechik et al., 1978). Their function, like that of mouse TL antigens, has not been determined.

The study reported here describes the fractionation by specific polysome immunoprecipitation of mRNAs specifying TL antigens. The proteins formed in the presence of TL-mRNA in a cell-free synthesizing system derived from wheat germ are larger than those present on the surface membranes of TL(+) cells.

Experimental Procedures

Cells and Isotope-Labeling Procedures. ASL-1 leukemia cells (TL 1,2,3), an in vitro maintained TL(+) murine leukemia cell line, were obtained from J. Buxbaum. They were cultured in 7% CO_2 at 37 °C in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics (growth medium). Cellular RNA was labeled during incubation of 10^7 cells/mL in growth medium to which 20 μCi/mL [^{3}H]-uridine (55 Ci/mmol, Amersham) had been added. Cellular proteins were labeled with [^{35}S]methionine (167 mCi/mmol, Amersham). Approximately 10^7 cells/mL were washed at 4 °C with methionine-free RPMI-1640 medium and then suspended in the same medium to which [^{35}S]methionine (250 μCi/mL) had been added. Incubation was performed at 37 °C for 1 or 5 h. In some experiments "exposed" membrane-associated proteins were labeled with ^{125}I according to procedures described by Wilder et al. (1979).

Preparation of TL 1,2,3 Antiserum and Purification of Immunoglobulin G. TL 1,2,3 antiserum was raised in A/J TL(−) congenic mice (the original breeding stock was obtained from E. A. Boyse) injected intraperitoneally at weekly intervals over a 10-week period with ~4.5 × 10^7 thymocytes from A/J TL(+) mice (Boyse et al., 1968a). Methods to determine

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[1] Abbreviations used: TL, thymus leukemia; mRNA, messenger ribonucleic acid; tRNA, transfer RNA; DEAE, diethylaminoethyl; CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; poly(A), poly(adenylic acid); oligo(dT), oligo(deoxythymidylate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; Cl_3AcOH, trichloroacetic acid; poly(U), poly(uridylic acid).

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antisera titers and their specificity were described previously (Cohen & Liang, 1976).

The γ-globulin fraction of TL 1,2,3, antiserum was obtained by ammonium sulfate precipitation (Garvey et al., 1977) followed by dialysis against 10 mM sodium phosphate and 15 mM NaCl buffer (pH 7.2). The redissolved precipitate was applied to a 1.0 cm diameter column containing 4 cm of DEAE- and 4 cm of CM-cellulose, both equilibrated with the same buffer as used for dialysis. The fraction obtained was concentrated by ultrafiltration and frozen. It had electrophoretic homogeneity in $NaDodSO_4$–polyacrylamide gels and was free of ribonuclease activity as judged by its lack of effect upon the structural integrity of polysomes of ASL-1 cells (Palacios et al., 1972). The titers of TL 1,2,3 antisera were 1:128 for most isolates.

Fractionation of TL Antigens of ASL-1 Cells by Extraction with Nonidet P-40. ASL-1 cells were treated with the nonionic detergent Nonidet P-40 (Particle Data Laboratories Ltd., Elmhurst, IL). Approximately 4×10^8 cells were suspended in 1 mL of 0.5% Nonidet P-40, 0.01 M Tris-HCl (pH 8.0), 0.15 M NaCl, 0.01 M EDTA, 0.02 N iodoacetamide, and 0.001 M phenylmethanesulfonyl fluoride (TBS buffer) and incubated for 30 min at 0 °C. Insoluble debris and large aggregates were removed by centrifugation at 10000*g*, followed by filtration through 0.45 μm Millipore filters.

TL antigens were obtained by indirect immunoprecipitation using TL 1,2,3, antiserum and rabbit anti-mouse immunoglobulins (RAM-IgG). 100 μL of cell lysate was incubated with 50 μL of TL antiserum for 2 h (0 °C), followed by second incubation for 1 h with 150 μL of RAM-IgG. Immunoprecipitates were washed with TBS buffer containing 0.2% Nonidet P-40.

Treatment of TL Antigens with Endo-β-N-acetylglucosaminidase H. Antigen–antibody precipitates dissociated during incubation at 80 °C for 5 min in 1% $NaDodSO_4$ and 50 mM Tris-HCl (pH 6.8). After centrifugation at 10000*g* for 15 min, the supernatant was diluted with an equal volume of 0.15 M sodium citrate buffer (pH 5.5). Endo-β-*N*-acetylglucosaminidase H, dissolved in 10 mM potassium phosphate buffer (pH 8.0) (concentration 100 μg/mL), was added to a final concentration of 5 μg/mL, and the samples were incubated for 2 h at 31 °C. Following incubation, $^1/_{10}$ volume of 10% $NaDodSO_4$ was added; the samples were heated to 80 °C for 5 min, and the macromolecules present were recovered by precipitation with 4 volumes of acetone (0 °C).

Isolation of Polysomes of ASL-1 Cells. Approximately 5×10^9 ASL-1 cells in log phase growth that had been labeled previously with [^{3}H]uridine (20 μCi/mL, 37 °C, 60 min) were washed with phosphate-buffered saline (pH 7.4) and suspended in 1.0 mL of TNMSH-100 buffer (25 mM Tris-HCl, pH 7.6, 25 mM NaCl, 5 mM $MgCl_2$, 0.5 M sucrose, and 100 μg/mL heparin); cell lysis occurred upon the addition of 100 μL of freshly prepared detergent solution consisting of 15% Triton X-100 and 5% sodium deoxycholate. The suspension was mixed vigorously, then incubated for 15 min at 0 °C, and centrifuged at 1000*g* for 5 min at 0 °C. The supernatant was transferred to 15-mL centrifuge tubes, mixed again with 100 μL of detergent solution, and centrifuged at 25000*g* for 15 min at 0 °C. The postmitochondrial supernatant was decanted and layered over a discontinuous sucrose gradient containing 2 mL of 1 M sucrose and 0.5 mL of 2.5 M sucrose, both in TNMH-500 buffer (25 mM Tris-HCl, pH 7.6, 25 mM NaCl, 5 mM $MgCl_2$, and 500 μg/mL heparin). Centrifugation was performed in a Spinco SW 60 rotor at 480000*g* for 45 min at 1 °C. After centrifugation, the opalescent polysome band at the boundary of two sucrose layers was removed with a sterile syringe through a hole introduced into the side of the tube. The polysomes were diluted with buffer (25 mM Tris-HCl, pH 7.1, 0.15 M NaCl, 5 mM $MgCl_2$, and 500 μg/mL heparin) to a final volume of 1 mL and dialyzed against the same buffer for 3 h at 0 °C.

Immunoprecipitation of Polysomes Forming TL Antigens. The procedure followed for immunoprecipitation of polysomes forming TL antigens is described in the footnote accompanying Table I.

Preparation of RNA from Immunoprecipitated Polysomes. Immunoprecipitated polysomes were suspended in 4 mL of TNMH-500 buffer containing 0.5% $NaDodSO_4$. An equal volume of liquified freshly distilled phenol was then added, and the mixture shaken was for 15 min. After centrifugation at 10000*g* for 5 min, the aqueous phase was reextracted once with an equal volume of phenol and twice with phenol–chloroform–isoamyl alcohol (25:24:1). One-tenth volume of 2.0 M sodium acetate was added to the aqueous phase, and the RNA was precipitated with 3 volumes of ethanol (−20 °C). The precipitate was collected by centrifugation at 10000*g* for 15 min and washed twice with 70% ethanol and then 3 times with 5 mL of 3 M sodium acetate (pH 7.0).

Poly(A)-containing RNA [poly(A)-RNA] was separated from other polysomal RNAs by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972).

Translation of TL-mRNA in a Cell-Free Protein Synthesizing System Derived from Wheat Germ. Protein synthesis directed by TL-mRNA obtained from ASL-1 cells was investigated in a cell-free protein synthesizing system derived from wheat germ. RNA (0.5 μg) was dissolved in 10 μL of H_2O and added to 40 μL of a reaction mixture containing 10 μL of buffer (100 mM Hepes, pH 7.0, 400 mM KCl, 12 mM magnesium acetate, and 5 mM 2-mercaptoethanol), 10 μL of solution containing 5 mM ATP, 125 μM GTP, 40 mM creatine phosphate, and 25 μg/mL creatine phosphokinase, 10 μL of S-30 wheat germ extract, 5 μL of all 19 unlabeled amino acids, and 5 μL of [^{3}H]leucine (2 μCi/μL, 55 Ci/mmol, Amersham). Samples were incubated for 3 h at 23 °C. Total radioactivity incorporated into the newly formed protein during synthesis was determined by Cl_3AcOH precipitation of 5-μL aliquots (Roberts & Peterson, 1973; Chan et al., 1976).

Results

Immunoprecipitation of Polysomes Forming TL Antigens. Approximately 1.6% of the polysomes of ASL-1 cells precipitated during incubation with purified mouse TL 1,2,3 antiserum, followed by the addition of an immunologic equivalent of rabbit anti-mouse IgG (Table I). (Also see paragraph at end of paper regarding supplementary material.) Substituting normal mouse IgG, normal mouse IgG that had been incubated previously with ASL-1 cells, rabbit anti-mouse IgG alone, or swine anti-rabbit IgG for specific antiserum led to the precipitation of <0.4% of the polysomes present. Substituting polysomes from the livers of tumor-free TL(+) A/J mice failed to lead to polysome immunoprecipitation under similar conditions.

Analysis of TL-mRNA. Poly(A)-containing RNA was obtained from immunoprecipitated polysomes by fractionation in columns containing oligo(dT)–cellulose. Approximately 2.5% of the RNA obtained from specifically precipitated polysomes bound to oligo(dT)–cellulose. The poly(A)-containing RNA ($A_{260}/A_{280} > 2.0$) recovered from an oligo(dT)–cellulose column was passed through a second column under similar conditions. The average specific activity of RNA

Table I: Immunoprecipitation of Polysomes from ASL-1 and Liver Cells[a]

polysomes	antibody	% of total cpm precipitated
ASL-1	normal mouse IgG	0.4
	normal mouse IgG preincubated with ASL-1 cells	0.3
	rabbit anti-mouse IgG	0
	mouse TL 1,2,3 antiserum	1.6
	swine anti-rabbit IgG	0
liver	normal mouse IgG	0
A/J (TL+) mice	mouse TL 1,2,3 antiserum	0

[a] Approximately 5×10^9 ASL-1 cells (10^7 cells/mL) were incubated at 37 °C for 60 min in RPMI-1640 medium supplemented with 10% fetal calf serum, antibodies, and 20 μCi/mL [^{3}H]uridine (55 Ci/mmol, Amersham) before the fractionation of polysomes. The polysomes were centrifuged at 25000*g* for 10 min at 0 °C immediately before the addition of specific antiserum. The supernatant was transferred to a 4-mL tube; 90 μL of the purified TL 1,2,3 antiserum (concentration 1.8 mg/mL) or normal mouse IgG (10 μL, 9.0 mg/mL) was added to the polysome suspension (10–70 A_{260} units). The mixture was incubated 30 min at 0 °C before an immunologic equivalent of rabbit anti-mouse IgG (350 μL, 1.0 mg/mL) was added, and the incubation was continued for an additional 30 min. As an additional control the polysomes were incubated as described with swine anti-rabbit immunoglobulin serum (200 μL, 1.5 mg/mL). The immunoprecipitate was layered over a discontinuous sucrose gradient consisting of 2 mL of 0.5 M sucrose and 4 mL of 1.0 M sucrose in TNMH-500 buffer containing 1% Triton X-100 and 1% sodium deoxycholate. It was centrifuged for 15 min at 25000*g* at 4 °C. The precipitate was resuspended in 1 mL of TNMH-500 and recentrifuged as above. The proportion of polysomes precipitated with specific antiserum was determined by comparing the radioactivity in immunoprecipitated polysomes with the radioactivity present in 1 equiv of polysomes precipitated with Cl_3AcOH.

bound to the column (TL-mRNA) was 4×10^5 cpm/A_{260} unit.

Poly(A)-containing RNA recovered from polysomes of ASL-1 cells precipitated with TL 1,2,3 antiserum was subjected to sucrose gradient centrifugation. The TL-mRNA fractionated from specifically immunoprecipitated polysomes possessed one identifiable size component sedimenting at ~7 S (Figure 1), corresponding to molecules of $\sim 7 \times 10^5$ daltons.

Polyacrylamide gel electrophoresis of immunoprecipitated polysomal RNA revealed the presence of 28S, 18S, and 4S RNAs, similar to that found for polysomes of ASL-1 cells obtained by conventional means.

To increase the sensitivity of detection of small quantities of TL-mRNA, we hybridized poly(A)-containing RNA from polysomes precipitated with TL 1,2,3 antiserum with [^{3}H]-poly(U); the hybrid was analyzed by sucrose gradient centrifugation (Figure 1) and polyacrylamide gel electrophoresis (Figure 2). The hybrid formed migrated in polyacrylamide gels as a single peak of radioactivity corresponding to an *s* value of 17–18. Electorphoresis of [^{3}H]poly(U) alone showed that poly(U) had an electrophoretic mobility lower than tRNA.

Estimation of the Molecular Weights of TL Antigens Formed in a Cell-Free System. Poly(A)-containing RNA obtained from polysomes of ASL-1 cells immunoprecipitated with TL 1,2,3 antiserum stimulated the synthesis in a cell-free system derived from wheat germ of products which themselves were specifically immunoprecipitated by TL 1,2,3 antiserum (Table II). Approximately 25% of the radioactivity incorporated into acid-precipitable material was precipitable by TL 1,2,3 antiserum. The proportion of radioactively labeled products recovered in this manner ranged in different experiments from 16.3% to 35.1%. Under similar conditions, <0.3%

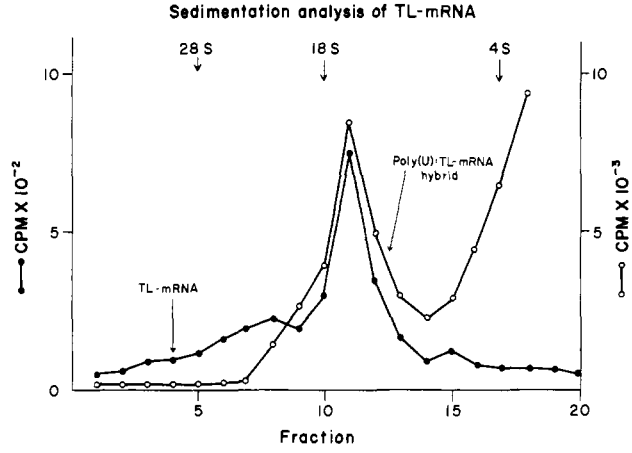

FIGURE 1: Sedimentation analysis of TL-mRNA. TL-mRNA isolated from immunoprecipitated polysomes was dissolved in 0.2 mL of buffer (10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$, and 0.1% $NaDodSO_4$), heated at 70 °C for 3 min, rapidly cooled, and layered over 3.8 mL of a 10–30% linear sucrose gradient (in the same buffer) containing 0.5% $NaDodSO_4$. Samples were centrifuged in a SW 60 rotor at 370000*g* for 140 min at 25 °C. After centrifugation, 0.2-mL fractions were collected, and the radioactivity present was determined after addition of Aquasol (New England Nuclear) in a liquid scintillation spectrometer. (●) TL-mRNA (0.01 A_{260} units, 4400 cpm/μg; (○) TL-mRNA (0.01 A_{260} units) incubated prior to centrifugation for 30 min with 0.03 μg of [^{3}H]poly(U) (500 mCi/mmol, 20000–30000 daltons).

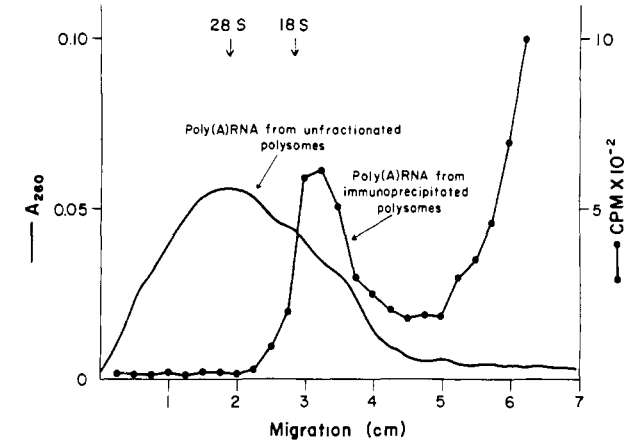

FIGURE 2: Polyacrylamide gel electrophoresis of TL-mRNA. RNA was dissolved in 40 μL of buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM Na_2EDTA, and 0.2% $NaDodSO_4$, pH 7.6) containing 14% sucrose and was fractionated in 2.4% polyacrylamide gels (0.6 × 7.0 cm) at 5 mA/gel. For electrophoretic analysis of [^{3}H]poly(U)–TL-mRNA hybrids, RNA was mixed with 0.01 μg of poly(U), and the mixture was incubated at 25 °C for 30 min. After electrophoresis the gels were scanned at 265 nm in a Gilford spectrophotometer, frozen at −20 °C, and sliced onto 2-mm sections. The slices were treated with 1 mL of NH_4OH (1 M), heated for 3 h at 65 °C, dried, swollen in 0.5 mL of water, and counted in 10 mL of Aquasol in a liquid scintillation spectrometer. (—) Total poly(A)-RNA from ASL-1 cells (0.04 A_{260} units); (●) poly(A)-RNA from immunoprecipitated polysomes hybridized to poly(U) (0.005 A_{260} units).

of the radioactivity incorporated in the presence of globin mRNA into acid-precipitable material was immunoprecipitable by TL 1,2,3 antiserum (Table II). Prior absorption of the TL 1,2,3 antiserum used by ASL-1 cells reduced the counts per minute immunoprecipitated to ~2.9% (Table II).

Gel electrophoresis of the specific precipitate obtained showed the preferential precipitation of a major protein with a M_r of 42000 (Figure 3). In contrast, no radioactive component was precipitated by serum from mice which were not specifically immunized.

Table II: Immunoprecipitation of the Products Synthesized in a Cell-Free System Derived from Wheat Germ[a]

RNA	cpm incorporated	cpm precipitated by TL 1,2,3 antibody	% of total cpm precipitated
poly(A)-RNA[c]	98 000 ± 6000	1960 ± 100	2.0 ± 0.6
TL-mRNA	45 000 ± 4000	11 000 ± 910	25.5 ± 4.3
globin mRNA	150 000 ± 9000	300 ± 46	0.2 ± 0.03
none	7000 ± 450	0	0
TL-mRNA	45 000 ± 4000	1100 ± 520[b]	2.4 ± 1.2

[a] 10 μL of mouse TL 1,2,3 antiserum of concentration 1.8 mg/mL and 240 μL of phosphate-buffered saline (pH 7.4) containing 1% sodium deoxycholate and 0.5% Triton X-100 were added to 50 μL of the protein synthesizing mixture. After incubation for 16 h at 4 °C, the mixture was incubated for 4 h more after the addition of 70 μL of RAM-IgG (1.0 mg/mL). The immunoprecipitates were purified by centrifugation through a 1 M sucrose "pad" and washed 3 times with phosphate-buffered saline containing sodium deoxycholate and Triton X-100. [b] The protein synthesizing mixture directed by TL-mRNA was incubated with TL 1,2,3 antibodies that were absorbed previously with ASL-1 cells. Nonspecific precipitation of products of translation directed by TL-mRNA was performed by using normal mouse IgG and rabbit anti-mouse IgG. Approximately 530 cpm were found in the immunoprecipitate of sample containing 48 000 of total cpm. [c] Poly(A)-RNA was isolated from unfractionated polysomes of ASL-1 cells.

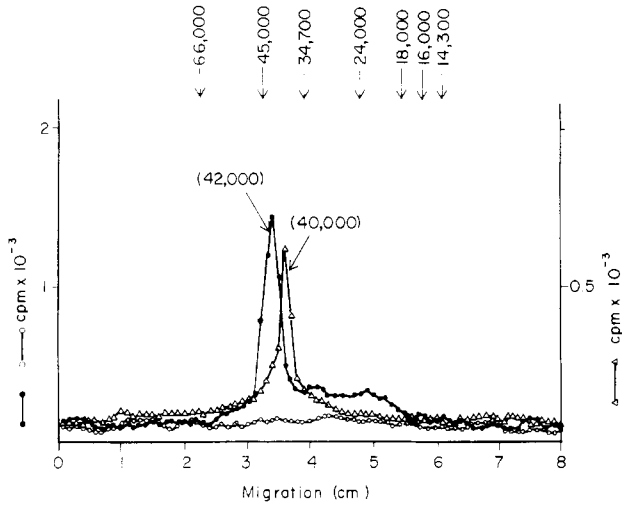

FIGURE 3: Polyacrylamide gel electrophoresis of the translation products. Purified samples of products of translation in vitro were precipitated with 5% Cl_3AcOH, washed twice with 2% Cl_3AcOH and twice with acetone, drained dry, and then resuspended in 40 μL of sample buffer (0.1 M sodium phosphate buffer, pH 7.0, 1% Na-$DodSO_4$, and 6 M urea). Samples were fractionated in 5% polyacrylamide gel (0.4 × 8.0 cm) prepared in 0.2 M phosphate buffer containing 0.2% Na$DodSO_4$, and electrophoresis was carried out at 5 mA/gel. After electrophoresis gels containing markers were stained with Coomsassie brillant blue, destained electrophoretically, and scanned at 580 nm in a Gilford spectrophotometer; gels containing protein samples were sliced into 1.0-mm sections, dissolved in 0.1 mL of 30% H_2O_2, and counted in 10 mL of Aquasol in a liquid scintillation spectrometer. (●) Translation products immunoprecipitated with TL 1,2,3 antiserum and RAM-IgG; (○) translation products precipitated with normal mouse IgG and RAM-IgG; (△) nonglycosylated TL antigens immunoprecipitated from the cells incubated in the presence of tunicamycin (1 μg/mL). Cells were labeled with [^{35}S]methionine for 5 h, and TL antigens were precipitated by using the double antibody technique as described under Experimental Procedures.

Molecular Weights of Nonglycosylated Heavy Chains. TL antigens present on the surface membranes of ASL-1 cells consist of heavy chains containing a carbohydrate side chain and a smaller polypeptide of 12 000 daltons equivalent to β_2-microglobulin (Ostberg et al., 1975). The molecular weight of nonglycosylated heavy chains was estimated by fractionating TL antigens from whole cells incubated previously in medium

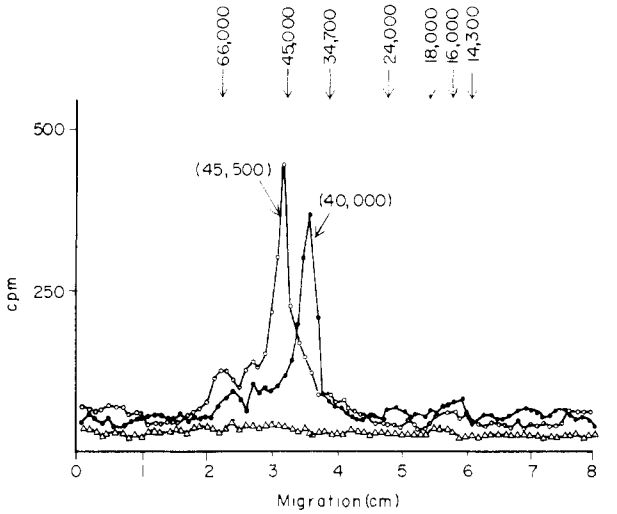

FIGURE 4: Digestion of TL antigens with endo-β-*N*-acetylglucosaminidase H. TL antigens were isolated by immunoprecipitation from lysates of ASL-1 cells labeled for 1 h with [^{35}S]methionine. (○) Intracellular TL antigens obtained by specific immunoprecipitation; (●) intracellular TL antigens obtained by specific immunoprecipitation and digested wtih endo-β-*N*-acetylglucosaminidase H; (△) cellular proteins precipitated with normal mouse immunoglobulins and RAM-IgG.

containing [^{35}S]methionine and tunicamycin, an inhibitor of N-linked glycosylation (Tkacz & Lampen, 1975). TL antigens were isolated by specific immunoprecipitation from Nonidet P-40 cell lysates of ASL-1 cells exposed previously for 5 h to 1 μg/mL tunicamycin. Immunoprecipitates were solubilized as described, and the molecular weights of the fractionated TL antigens were estimated by electrophoresis in polyacrylamide gels. A predominant single peak was observed with a molecular weight of ~40 000 equivalent to heavy chains of TL antigens. No radioactivity peak corresponding to β_2-microglobulin was observed (Figure 3).

The molecular weight of nonglycosylated heavy chains was also estimated by treating immunoprecipitated TL antigens with endo-β-*N*-acetylglucosaminidase H (Tarentino & Maley, 1974). In these experiments cells were labeled for 1 h with [^{35}S]methionine and extracted with Nonidet P-40. The heavy chains of TL antigens were similar to those observed for antigens fractionated from cells incubated in medium containing tunicamycin (Figure 4). The molecular weight of the heavy chains of TL antigens fractionated and separated in polyacrylamide gels without enzyme treatment was 45 500. These data may reflect intracellular events occurring during the process of cellular biosynthesis of TL heavy chains.

For determination of the molecular weight of heavy chains expressed by the cells, i.e., present on the surface membranes of TL(+) cells, ASL-1 cells were labeled with ^{125}I and extracted with Nonidet P-40. TL antigens were immunoprecipitated with specific antiserum. Electrophoresis of radioiodinated samples yielded a TL antigen heavy chain peak of M_r 47 000 and smaller one of 12 000 equivalent to β_2-microglobulin (Figure 5). Equivalent results were obtained when TL antigens were immunoprecipitated from cells labeled for 1 h with [^{35}S]methionine and "chased" for 3 h in a medium containing cold methionine.

Discussion

The data reported here indicate that the nonglycosylated heavy chains of TL antigens of murine leukemia cells possess a molecular weight of ~40 000. Two lines of experimental evidence support this conclusion. Treatment of isolated heavy chains of TL antigens of murine leukemia cells with the en-

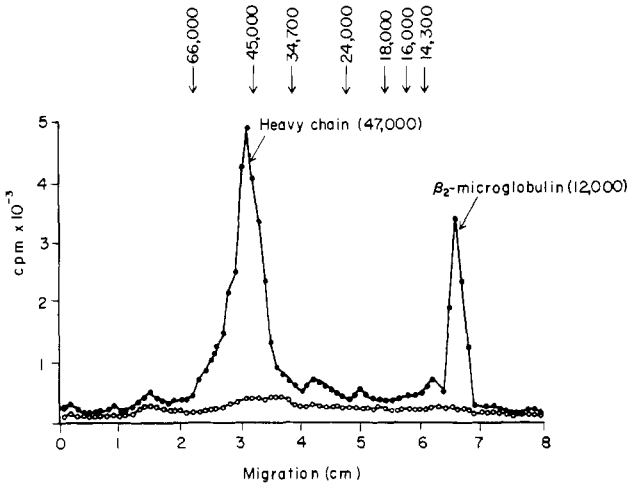

FIGURE 5: Polyacrylamide gel electrophoresis of ^{125}I-labeled cell surface antigens. Surface antigens of ASL-1 cells were radioiodinated with the lactoperoxidase technique, extracted with nonionic detergent, and immunoprecipitated by using TL 1,2,3 antiserum (●) or normal mouse IgG (○).

zyme endo-β-*N*-acetylglucosaminidase H, specific for the high-mannose type of oligosaccharide side chain, yields a single peak in polyacrylamide gel electrophoresis of this size equivalent. Fractionation of heavy chains of TL antigens from cells incubated previously in medium containing tunicamycin, an antibiotic inhibitor of de novo glycosylation, yields a polypeptide of similar molecular size. The molecular weight of glycosylated heavy chains at the membrane is 47 000.

The methods used for fractionating TL antigens have been applied previously to investigate various parameters of their structure and cellular physiology. The native structure of TL antigens is composed of a heavy chain possessing the determinants of the TL antigen complex along with a light chain equivalent to β_2-microglobulin. The heavy–light chain dimer may exist at the plasma membrane as a tetramer consisting of two heavy and two light chains in a four-chain unit. Association of the complex with the membrane is through the C-terminal portion of the heavy chain (Ostberg et al., 1975). Similar methods of fractionation have been used to fractionate and characterize membrane-associated H-2 antigens and immunoglobulin-like determinants on murine cells. These determinants possess, as do TL antigens, heavy and light chains (Vitetta et al., 1976; Michaelson et al., 1977).

The method of fractionation of hydrophobic macromolecular antigens from cells is dependent upon the preservation of their antigenic structure in nonionic detergent solution. Previous experience with this method indicates that each of several antigenically distinct molecules may be immunoprecipitated selectively (Liang & Cohen, 1977; Vitetta et al., 1976), that the recovery is quantitative (Liang & Cohen, 1977), and that antiserum with specificity toward one determinant leads to the precipitation of others if they are associated with single complex macromolecules (Uhr et al., 1976).

Polysomes forming TL antigens precipitate as immune complexes in the presence of TL antiserum. The antiserum used possessed reactivity for TL 1,2,3 antigenic determinants. It was raised in TL(−) congenic strain A mice immunized with thymocytes from TL(+) mice of the same mouse strain. The only known antigenic disparity between the cells used as immunogens and those of recipients is present in the TL antigen complex. Analysis of detergent-extracted ASL-1 cells by $NaDodSO_4$–polyacrylamide gel electrophoresis of immunoprecipitates prepared with such antiserum indicates that the antiserum is monospecific. No demonstrable reactivity for other determinants of ASL-1 cells is detectable by immunologic criteria in the antiserum used. Specific immunoprecipitation of polysomes has been used successfully to fractionate polysomes forming rat albumin (Shapiro et al., 1974), hen oviduct ovalbumin (Shapiro et al., 1974; Palacios et al., 1972), and mouse immunoglobulins (Schechter, 1975; Legler & Cohen, 1976).

It is conceivable that immunologic methods are insufficiently sensitive and/or precise to reveal the presence in the antiserum used of antibody specificities toward non TL associated determinants of ASL-1 cells. Although evidence by immunologic criteria indicates that the antiserum used is monospecific, non TL antigen binding proteins present may have led to the precipitation of products formed in the wheat germ system which are distinct from those formed by ASL-1 cells.

TL antigens, hydrophobic membrane-associated macromolecules, are soluble in detergent solution. Immunoprecipitation of polysomes was performed in the presence of Triton X-100 and sodium deoxycholate. The precise intracellular hydrophobic environment present to meet the specialized needs of the cell in forming membrane-associated macromolecules has not been described in detail.

The TL-mRNA obtained from immunoprecipitated polysomes migrates during sucrose density gradient centrifugation or in $NaDodSO_4$–polyacrylamide gel electrophoresis as a single predominant band corresponding to ~17 S. Precise estimations of its molecular weight would be premature since uncontrolled variables, e.g., its secondary structure, affect its migration rate under these circumstances.

RNA preparations were obtained in quantities sufficient for the translation in vitro. Heavy chains of TL antigens synthesized in vitro from such mRNAs were 2000 daltons larger than their respective nonglycosylated molecules obtained from ASL-1 cells. This suggests that an "extra" peptide of ~20 residues is present, in agreement with molecular weight estimates and sizes of NH_2-terminal extensions found previously for several proteins of eukaryotic cells (Devillers-Thiery et al., 1975), including immunoglobulins (Burstein & Schechter, 1978) and a virally specified membrane glycoprotein (Lingappa et al., 1978). It is conceivable that membrane-associated macromolecules like those secreted from the cells contain a presequence termed a "signal peptide" by Blobel and coworkers (Blobel & Dobberstein, 1975a,b). Signal peptides are characteristically hydrophobic (Blobel & Dobberstein, 1975a). Mandel & Wickner (1979) have described a "leader peptide" in the coat protein of coliphage M13, postulated to be involved in maintaining protein solubility in a hydrophilic environment.

The rate of processing of the putative 42 000 molecular weight heavy-chain precurser has not been determined. The fact that it was not found in extracts of ASL-1 cells suggests that it does not accumulate in amounts sufficient for detection by the methods we used. Conceivably, the "extra" polypeptide is processed shortly after it is synthesized.

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Supplementary Material Available

Sequential immunoprecipitation of TL antigens and cytotoxic effects of TL 1,2,3 antiserum (2 pages). Ordering information is given on any current masthead page.

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