ABSTRACT

We have investigated the number of structural genes present in ASL-1 cells, a murine leukemia cell line which encodes the heavy chain of thymus leukemia (TL) antigens, a protein which is similar to the Class I histocompatibility antigens. TL-specific messenger RNA was purified from polysomes of ASL-1 cells by immunoprecipitation, and this messenger RNA translated in vitro to produce a M, 42,000 protein which comigrated on acrylamide gel with nonglycosylated TL heavy chain. A 32P-labeled complementary DNA (cDNA) was synthesized by reverse transcription of the TL-specific messenger RNA as template. Analysis of reassociation kinetics of the 32P-TL-cDNA with DNA from ASL-1 cells showed that the kinetics was indistinguishable from that obtained using a DNA encoding a single-copy gene (C/¿). An analysis was performed in which DNA from ASL-1 cells was subjected to digestion with each of three restriction enzymes and hybridized with 32P-TL-cDNA according to the Southern "blot" technique. Two bands formed hybrids with the 32P-TL-cDNA with each of three restriction enzymes used. These data are consistent with the presence of a small number of structural genes for the TL heavy chain in the genome of ASL-1 leukemia cells.

INTRODUCTION

TL antigens are integral membrane glycoproteins associated with the surface membranes of immature thymus cells of some but not all mouse strains (10, 11). Thymus-derived cells in the spleen and other peripheral lymphoid organs spontaneously lose TL antigens as they mature into helper or cytotoxic T-lymphocytes. Murine leukemia of thymus origin may express TL antigens, reflecting their less differentiated state (3, 5).

On the cell surface, TL antigen molecules consist of 2 polypeptide chains: a glycosylated heavy chain (M, 47,000), which bears the determinants of the TL antigen complex; and β2-microglobulin, a "light chain" (M, 12,000). The heavy chain is an integral membrane protein; its carboxy terminus is imbedded in the membrane. The β2-microglobulin chain is covalently coupled to the heavy chain and is not directly associated with the membrane. The basic structure of the TL antigen complex is similar to that of the classical histocompatibility antigens and, as such, TL antigens are considered to be Class I molecules (7).

The one region of the mouse chromosome containing the locus coding TL comprises about 2000 kilobases of DNA (15).

MATERIALS AND METHODS

Cells and Isotope Labeling. ASL-1 cells of strain A mouse origin, an in vitro-maintained TL 1,2,3+ murine leukemia cell line, were obtained from J. Buxbaum (New York University, New York, N. Y.). They were cultured in 7% CO₂ at 37° in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum (growth medium). Cellular RNA was labeled during incubation of 10⁷ cells/ml in growth medium supplemented with [¹H]uridine (20 μCi/ml; 55 Ci/mmol; Amersham, Arlington Heights, Ill.).

Preparation of TL-specific mRNA. Polysomes were isolated by sucrose gradient centrifugation of cytoplasmic extracts of ASL-1 cells as described (13). Polysomes which contained mRNA encoding TL antigens were immunoprecipitated with TL 1,2,3 antiserum and were purified by sucrose gradient centrifugation as described (13). TL 1,2,3 antiserum was raised in strain A TL (-) congenic mice (bred in our animal colony; the original breeding pair was obtained as a gift from E. A. Boyse, Memorial Hospital, New York, N. Y.) given injections of thymus cells from strain A mice (5). Polyclonal RNA was isolated from the immunoprecipitated polysomes by chromatography on oligodeoxynucleotide-cellulose (1). Approximately 2.5% of the total polysomal RNA bound to the column. The 17S fraction of poly(A)⁺RNA was isolated by passage over a 10 to 30% linear sucrose gradient as described (13). After centrifugation, fractions corresponding to 17S were combined and precipitated with ethanol.

Translation of TL mRNA in Cell-free Systems Derived from Rabbit Reticulocytes. TL mRNA was translated in a rabbit reticulocyte lysate system (BRL, Bethesda, Md.). One μg of 17S poly(A)⁺RNA from immunoprecipitated polysomes of ASL-1 cells was added to 30 μl of reaction mixture. Proteins were labeled during translation with 5 μCi [¹H]leucine.
(Amersham); the incorporation of \(^{3}H\)leucine was measured by determining the amount of radioactivity that was precipitated with trichloroacetic acid. The translation products were precipitated with specific antiserum and subjected to SDS-polyacrylamide gel electrophoresis in 7% acrylamide (9).

**Synthesis of TL-cDNA.** TL-cDNA was synthesized using reverse transcriptase in the presence of \(^{32}P\)-labeled nucleotides. The reaction was carried out as described by Efstratiadis et al. (6). The total incorporation of \(^{32}P\) was 3 x 10^5 cpm. The TL-cDNA was then fractionated by 1% agarose gel electrophoresis in 0.04 M Tris-acetate (pH 7.4)-0.002 M disodium EDTA-0.01 M NaCl buffer. After electrophoresis, the gel was subjected to autoradiography for 2 hr, and a well-defined single band of a size equivalent to TL mRNA was excised from the gel. The cDNA was eluted from agarose gel with 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA, and 0.1% SDS for use in hybridization studies.

**Isolation of High-Molecular-Weight DNA from ASL-1 Cells.** Approximately 5 x 10^6 ASL-1 cells were suspended in 10 ml of buffer [0.14 M NaCl-10 mM Tris-HCl (pH 7.4)-1.5 mM MgCl\(_2\)] containing 0.5% Nonidet P-40 and incubated for 15 min at 0°. Nuclei were collected by centrifugation at 1000 x g and suspended in protease K buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA-10 mM NaCl-0.5% SDS-protease K (100 \(\mu\)g/ml); Beckman, Schiller Park, Ill.]. The nuclear lysate was incubated for 24 hr at 37° and then was extracted twice with phenol and precipitated with ethanol. The DNA was then purified by cesium chloride gradient centrifugation.

**Digestion of DNA from ASL-1 Cells with Restriction Endonucleases.** Restriction endonuclease, EcoRI, BamHI, and HindIII, were obtained from BRL. Nuclear DNA isolated as described above was digested using standard conditions, and the resulting fragments were fractionated by agarose gel electrophoresis. Restriction fragments of bacteriophage \(\lambda\) DNA were used as size markers. After electrophoresis, the DNA fragments were transferred onto nitrocellulose paper (14) and were hybridized with \(^{32}P\)-TL-cDNA.

**Analysis of Reassociation Kinetics.** Hybridizations were carried out using a mixture of 10 \(\mu\)g DNA from ASL-1 cells, about 2 ng of \(^{32}P\)-TL-cDNA, and poly(A)*RNA (0.1 mg/ml) in a total volume of 0.5 ml of a buffer containing 0.6 M NaCl, 40 mM Tris-HCl (pH 7.4), and 2 mM EDTA. The samples were incubated at 100° for 10 min and were then transferred to 68° and incubated for varying periods (2). The reactions were terminated by adding 0.5 ml of S, nuclease buffer and digesting single-stranded DNA with S, nuclease (Calbiochem, San Diego, Calif.) (9).

**RESULTS**

**In Vitro Translation of TL mRNA.** Poly(A)*RNA obtained from immunoprecipitated polysomes of ASL-1 cells stimulated the synthesis in a cell-free system derived from rabbit reticulocytes of protein products which were specifically immunoprecipitated by anti-TL 1,2,3 antiserum (Table 1). Approximately 40% of the radioactivity incorporated into acid-precipitable material was precipitated by anti-TL 1,2,3 antiserum, whereas only 0.3% of radioactivity was precipitated by the antiserum when globin mRNA was used as message in this system. Electrophoresis in polyacrylamide gels of the immunoprecipitated translation products revealed the presence of a protein with a molecular weight of 42,000 equivalent to nonglycosylated TL heavy chain containing a signal peptide (13) (Fig. 1). No other radiolabeled proteins were detected in the immunoprecipitates.

**Table 1**

<table>
<thead>
<tr>
<th>RNA</th>
<th>cpm Incorporated</th>
<th>cpm Precipitated by TL 1,2,3 antibody</th>
<th>% of Total cpm precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL-mRNA</td>
<td>79,000 ± 8,000</td>
<td>32,000 ± 1,800</td>
<td>40.5 ± 5.7</td>
</tr>
<tr>
<td>Globin</td>
<td>220,000 ± 12,000</td>
<td>620 ± 95</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>mRNA</td>
<td>12,000 ± 800</td>
<td>40 ± 20</td>
<td>0.3 ± 0.05</td>
</tr>
</tbody>
</table>

*Mean ± S.D.*

No other bands were detected in the immunoprecipitates.
Our earlier work demonstrated that mRNA isolated from unfractionated polysomes of ASL-1 cells sedimented in sucrose gradients as a 17S species (13). In vitro translation products of the 17S RNA produced a M, 42,000 protein identical in polyacrylamide gels to that produced from the anti-TL-polyribosomal polysomes of ASL-1 cells (Fig. 1). In control experiments, no radioactive components were precipitated by sera from mice which were not specifically immunized, by antisera of specificity other than for the TL 1,2,3 antigens or by TL 1,2,3 antisera absorbed prior to use with thymocytes from A/J mice.

Preparation of TL-Specific cDNA. Single-stranded DNA complementary to poly(A)+ RNA from specifically immunoprecipitated polysomes was synthesized in the presence of [32P]-labeled nucleotides. In agarose gels, the predominant species of [32P] cDNA comigrated with the 17S TL-specific RNA (Fig. 2). Other DNA molecules of smaller size, probably representing partial reverse transcripts of TL-mRNA, were present in lesser amounts.

Estimation of Copy Number of TL Genes by Analysis of Reassociation Kinetics. To determine whether genes specifying TL antigens are present in single or multiple copies in the DNA of ASL-1 mouse leukemia cells, we determined the kinetics of reassociation of DNA from ASL-1 cells with [32P]-TL-cDNA. A cDNA clone, µ12, containing a portion of the single-copy gene encoding the heavy chain of mouse IgM (4), was used as a calibration standard. The kinetics of reassociation of [32P]-TL-cDNA with DNA from ASL-1 cells was indistinguishable from that of the single-copy control gene (Chart 1). For both, the Cfo value obtained was approximately 10^−3. Thus, according to this analysis, the copy number of the gene specifying the TL antigen heavy chain in ASL-1 cells is approximately equivalent to the number of genes specifying the constant region of the mouse IgM heavy chain in the same cell type.

Hybridization of [32P]-TL-cDNA to Immobilized DNA from ASL-1 Cells. To visualize directly the number of DNA fragments in ASL-1 cells hybridizing with [32P]-TL-cDNA, DNA from ASL-1 cells was digested with restriction endonucleases, electrophoresed in agarose gel, and transferred onto nitrocellulose paper. The immobilized DNA was then subjected to hybridization with [32P]-TL-cDNA. At least 2 clearly distinct DNA fragments produced by each of the restriction endonucleases used hybridized strongly with the [32P]-TL-cDNA. Variation in the intensity of the bands was noted (Fig. 3), suggesting that some bands represented fragments containing only a short sequence homologous to the [32P]-TL-cDNA probe or that some bands contained more than one comigrating fragment containing sequences homologous to the probe.

DISCUSSION

The mRNA-specifying heavy chain of the TL antigens has been isolated and used as template to synthesize a [32P]cDNA. This cDNA was used in 2 assays to estimate the number of genes encoding TL antigens in the genome of ASL-1 leukemia cells. These 2 assays, reassociation kinetics and hybridization to immobilized genomic DNA, both indicated that the number of TL-specifying genes is small, on the order of one or 2 copies per haploid genome.

The kinetics of reassociation between [32P]-TL-cDNA and DNA from ASL-1 leukemia cells was identical to that of a cloned cDNA encoding IgM heavy chains, a gene segment known to occur in a single copy in the haploid genome of the mouse. This analysis indicated that the gene encoding TL antigen heavy chains occurs as a single copy or as a very small number of related genes. It is difficult to distinguish a difference in reassociation kinetics between unique sequences and those occurring 2 or 3 times in a haploid genome in part because self-annealing of the DNA occurs during the prolonged incubation periods required to obtain high Cfo values.
Another estimate of the number of TL genes was obtained by hybridizing 32P-TL-cDNA with restriction digests of DNA from ASL-1 leukemia cells, according to the Southern blot technique. With each of the 3 restriction enzymes used, 2 distinct fragments formed detectable hybrids with 32P-TL-cDNA. Thus, results of the blot hybridization confirm the conclusion drawn from reassociation kinetics, namely, that DNA from ASL-1 cells contains only a small number of sequences homologous to the 32P-cDNA. We are not able to determine whether the 2 bands represent 2 distinct structural genes or a single structural gene containing sites for each of the 3 restriction endonucleases used. Nucleotide sequence analysis is required to establish the precise structure of the TL gene(s). The results of the hybridization studies reported here indicate that the 32P-TL-cDNA transcribed from purified TL mRNA by reverse transcriptase does not cross-hybridize with sequences encoding Class I transplantation antigens, under the conditions used.

Some variation in the intensity of bands was noted on autoradiograms of the genomic blots. Such variation can simply reflect an unequal efficiency of transfer of the DNA onto nitrocellulose, or it can indicate differences between bands in the amount of sequences homologous to the labeled probe. A difference in the extent of homology can indicate either that the enzyme cleaved the genomic DNA within the coding sequence to produce one fragment with only a short region of homology and one with the majority of the gene or that one band represents a second gene segment only partially homologous to the probe. A partially homologous gene segment could be a pseudogene which has lost part of the coding sequence by deletion, or it may be a functional gene encoding a second kind of TL heavy chain which has not been detected in studies at the cellular or protein level.

Interpretation of the results obtained here depends on the specificity of the 32P-TL-cDNA used. The cDNA was synthesized from a preparation of poly(A)+RNA isolated from polyribosomes which were immunoprecipitated with TL 1, 2, 3 antiserum. The 17S RNA species obtained directs translation of a protein product which comigrates in SDS-polyacrylamide gel electrophoresis with nonglycosylated TL heavy chains; other bands were not detectable. The antiserum used was prepared by immunizing TL-minus A/J congenic mice with thymocytes from TL-positive A/J mice: the only known antigenic disparity between TL-positive and TL-minus congenic strain A mice is associated with determinants present on the heavy chain of the TL-antigen complex. Although it is likely that heterogeneous mRNAs specifying other proteins were present in the preparations that we used, we were unable to detect a predominant contaminating species which would have complicated the interpretation of our results. Furthermore, the reassociation kinetics data and Southern blot results indicating a small number of genes present support the notion that a predominant species of RNA was present.

The predominant species of 32P-TL-cDNA comigrated on an agarose gel with the 17S RNA species shown by in vitro transcription to encode the TL heavy chain. Some smaller cDNA molecules were also present, probably representing partial reverse transcripts of the large TL mRNA (approximately 1100 base pairs estimated from the molecular weight of the protein). The absence of detectable weakly hybridizing bands on the genomic blot obtained indicates that it is unlikely that a single contaminating RNA species is present in sufficient amounts to interfere with interpretation of the experimental results. Cross-hybridization of the cDNA that we used with other genes specifying Class I histocompatibility antigens was not observed; the cDNA was prepared from mRNA for TL heavy chains specific only for a portion of the total genomic region involved. It appears, therefore, that a small number, as few as one or 2 genes, encodes the TL antigen heavy chain in DNA of ASL-1 mouse leukemia cells.

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**REFERENCES**

Estimation of the Number of Genes Specifying the Heavy Chain of Mouse Thymus Leukemia Antigens

Ryszard Slomski, Christine L. Martens and Edward P. Cohen


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