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Immunology

Cell-free synthesis of mouse H-2 histocompatibility antigens

(*H-2K* and *H-2D* loci/cell surface antigens/allograft rejection)

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ABSTRACT **The mRNAs coding for the histocompatibility (H-2) antigens of the mouse have been identified by cell-free translation of poly(A)-containing RNA obtained from the livers of mice (strain A/J), followed by immunoprecipitation of the cell-free products by using an antiserum directed against purified H-2^a. Unlike the 47,000- and 46,000-M_r H-2 glycoproteins synthesized in splenic lymphocytes, the cell-free translation products have M_rs of 45,000 and 44,500, representing the unglycosylated forms of these antigens. The cell-free products are shown to be related to the H-2 antigens by competition immunoprecipitation with purified H-2^a and by two-dimensional tryptic peptide mapping. The H-2 mRNAs which sediment at 17 S are found associated predominantly with membrane-bound polysomes and are actively translated in the liver where as many as 16 ribosomes are associated with each molecule of H-2 mRNA. The implications of these studies for molecular cloning and for an understanding of the organization and expression of the genes encoding these H-2 antigens are discussed.**

The major histocompatibility complex of the mouse controls a number of cellular properties important to the immune system (1). Genetic studies have mapped a series of loci that regulate some of these traits to specific regions within the complex. Two of these loci, *H-2K* and *H-2D*, determine the serologically detectable alloantigens present on all cells in the adult mouse. A knowledge of the molecular structure of these histocompatibility (H-2) antigens has long been sought as a key to understanding the functions of these molecules and the complex genetic system that controls them.

The H-2K and H-2D molecules show extensive homology in their primary structure (2–4). They are glycoproteins consisting of a single polypeptide chain to which two carbohydrate sidechains are attached (5). These H-2 molecules are anchored in the plasma membrane by a hydrophobic region at their carboxyl termini (6, 7). At the cell surface, the amino-terminal portion is found noncovalently associated with a molecule of β_2 microglobulin, a 12,000-M_r polypeptide encoded by a gene that is not part of the H-2 complex (8, 9).

Apart from their role in the restriction of the specificity of lymphocytes involved in the recognition of viral and tumor antigens (10), the H-2 molecules are responsible for determining compatibility in tissue and organ transplantation (1). The complexity of allograft rejection is the result of their genetic polymorphism. It has been estimated that there are at least 56 alleles at the *H-2K* locus and 45 alleles at the *H-2D* locus (11). This would allow some 2500 different combinations of alleles at these two loci alone; most of these combinations probably do exist in the population, because >90% of all mice are heterozygous at these loci (12).

Because the allelic products of these *H-2* loci differ by many amino acid substitutions, it is clear that they cannot be derived from a single mutation. At present, the origin of such extensive polymorphism at the *H-2* loci is obscure. One approach to this problem is to characterize the mRNAs that encode these H-2 antigens, as a means to providing some insight into the overall organization of these genes. We describe here the identification and partial purification of the H-2 mRNAs from mouse livers.

MATERIALS AND METHODS

Materials. A/J mice (*H-2^a* haplotype), 6–8 weeks old, were purchased from the Jackson Laboratory. Purification of papain-solubilized H-2^a antigen (*p*H-2^a) and characterization of the rabbit anti-H-2^a serum have been described (13). Ethyl[1-^{14}C]acetimidate (10 Ci/mol; 1 Ci = 3.7×10^{10} becquerels) and [^{35}S]methionine (1000 Ci/mmol) were purchased from Amersham. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was obtained from Worthington.

Labeling of Cells. Spleen cells from A/J mice were labeled with 50 μCi of [^{35}S]methionine per ml of Earle's balanced salt solution containing 5% minimal essential medium and 2% fetal bovine serum. After incubation for 2 hr at 37°C, the cells were washed and lysed with Tris-buffered saline containing 1% Nonidet P-40 and 2 mM phenylmethylsulfonyl fluoride.

Preparation of mRNA. Livers from A/J mice initially frozen in liquid N_2, were homogenized with equal volumes of extraction buffer [50 mM Tris·HCl, pH 9/0.1 M NaCl/1 mM EDTA/1% sodium dodecyl sulfate ($NaDodSO_4$)/0.1% heparin] and phenol/chloroform/isoamyl alcohol (50:50:1) in a Waring Blendor for 2 min at full speed. After two subsequent extractions with the phenol mixture, the aqueous phases were precipitated with cold ethanol at −20°C. Selection of poly(A)-containing RNA was performed on an oligo(dT)-cellulose column (14). The poly(A)-containing RNA, brought to 65°C for 5 min and rapidly chilled to 4°C, was fractionated further by centrifugation on a 5–20% (wt/vol) sucrose/0.1% $NaDodSO_4$ gradient.

Free and membrane-bound polysomes were prepared according to Ramsey and Steele (15). Polysomes were fractionated by centrifugation on a 15–40% (wt/vol) sucrose gradient. Poly(A)-containing RNA from each polysome fraction was selected as described above after adjustment to 0.5 M NaCl and 0.4% $NaDodSO_4$. RNA was solubilized in water at a concentration of about 1 mg/ml and stored at −70°C.

Cell-Free Protein Synthesis. Cell-free translation was carried out essentially as described by Pelham and Jackson (16) in the rabbit reticulocyte lysate system made mRNA-dependent by treatment with micrococcal nuclease.

Immunoprecipitation and $NaDodSO_4$/Polyacrylamide Gel Analysis. Immunoprecipitation with antiserum and heat-inactivated *Staphylococcus aureus* was performed as

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Abbreviations: *p*H-2, papain-solubilized H-2; $NaDodSO_4$, sodium dodecyl sulfate.

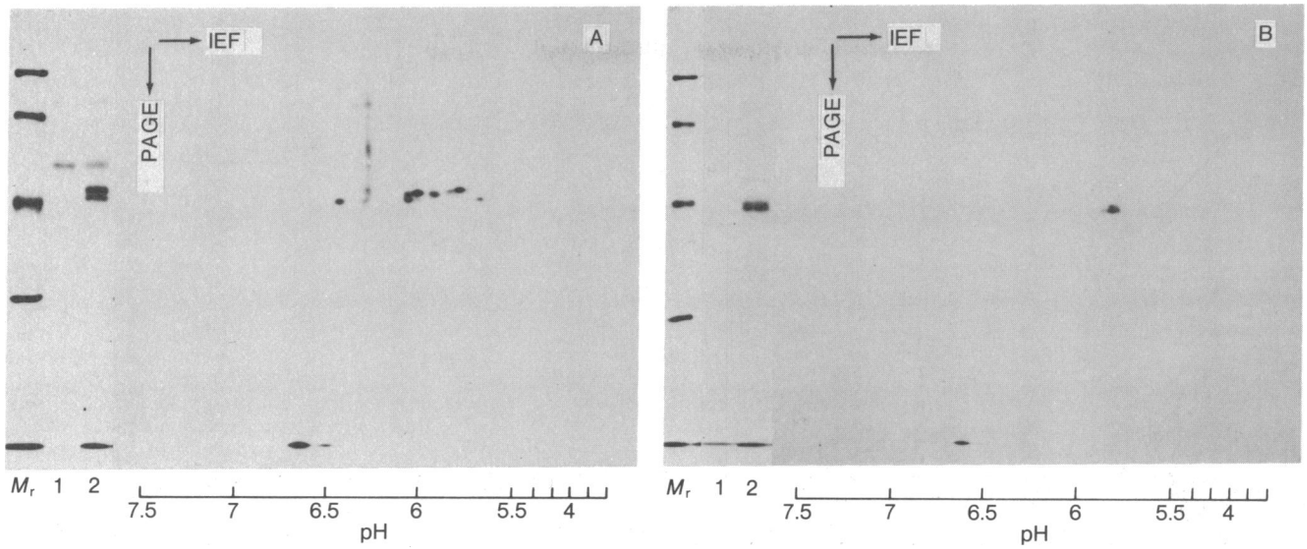

FIG. 1. Immunoprecipitation of H-2^a antigens synthesized either *in vivo* in splenic lymphocytes or *in vitro* in rabbit reticulocyte lysates. (*A*) One- and two-dimensional gel electrophoresis of [^{35}S]methionine-labeled immunoprecipitates from extracts of A/J splenic lymphocytes using either normal rabbit serum (lane 1) or rabbit anti-H-2^a serum (lane 2 and two-dimensional gel). (*B*) One- and two-dimensional gel electrophoresis of [^{35}S]methionine-labeled cell-free products immunoprecipitated from rabbit reticulocyte lysates programmed with poly(A)-containing RNA from livers of A/J mice, using either normal rabbit serum (lane 1) or rabbit anti-H-2^a serum (lane 2 and two-dimensional gel). The M_r markers used were phosphorylase b (92,500), bovine serum albumin (69,000), ovalbumin (45,000), carbonic anhydrase (30,000), and cytochrome *c* (12,300). PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

described (17). Immunoprecipitates were analyzed by electrophoresis in 10% polyacrylamide/NaDodSO$_4$ slab gels (18). Two-dimensional gel analysis was performed by isoelectric focusing in the presence of ampholine (pH 3.5–10), followed by NaDodSO$_4$/polyacrylamide slab gel electrophoresis (19). [^{35}S]Methionine-labeled proteins were detected by fluorography (20).

Tryptic Digestion, Radiolabeling of *p*H-2^a, and Peptide Mapping. Fully reduced and carboxymethylated *p*H-2^a heavy chain was digested with trypsin at a 1:25 (wt/wt) ratio. After incubation for 1.5 hr at 37°C, trypsin was added again and the incubation was continued for another 3 hr. The extent of cleavage was estimated by digestion of an aliquot with carboxypeptidase B followed by amino acid analysis (Durrum D500) for lysine and arginine. The lyophilized tryptic digest was mixed with 0.1 M ethyl[1-^{14}C]acetimidate (1.1 Ci/mol) in 0.2 M *N*-ethylmaleimide acetate, pH 8.2, and the reaction was carried out for 1 hr at 25°C (21). The reaction mixture was desalted on Sephadex G-10. Tryptic digestion of [^{35}S]methionine-labeled proteins fractionated on NaDodSO$_4$/polyacrylamide gels was performed in 0.1 M sodium bicarbonate at 37°C. The lyophilized tryptic peptides were resuspended in 15 mM NH_4OH and applied to cellulose thin-layer plates. Electrophoresis in the first dimension was carried out in 30% formic acid. Chromatography in the second dimension was performed at pH 6.0 in butanol/acetic acid/pyridine/water (60:12:40:48).

RESULTS

Immunoprecipitation of Products of the *H-2K* and *H-2D* Loci with an Antiserum Directed Against Purified H-2^a Antigen. An anti-H-2^a serum has been prepared by immunizing rabbits with a purified preparation of *p*H-2^a antigen from A/J mice (13). Because the antiserum is directed against total H-2^a antigen, it is expected to recognize the products of both the *H-2K* and *H-2D* loci, as well as β_2 microglobulin.

To identify the H-2 products, 1% Nonidet P-40 extracts of [^{35}S]methionine-labeled A/J splenic lymphocytes were immunoprecipitated with either normal rabbit serum or rabbit anti-H-2^a serum. Analysis of the resulting immunoprecipitates on a polyacrylamide/NaDodSO$_4$ slab gel (Fig. 1*A*) showed two components of M_rs 47,000 and 46,000 that were specifically precipitated by the anti-H-2^a serum (lane 2) but not by the normal serum (lane 1). Previous studies (22) suggest that the 47,000-M_r and 46,000-M_r components may represent products of the *H-2K* locus and the *H-2D* locus, respectively. Also precipitated by the anti-H-2^a serum was β_2 microglobulin, a 12,000-M_r component which migrated to the bottom of the gel. Two components with M_rs of 75,000 and 54,000 detected with both normal and anti-H-2^a serum were the heavy chains of IgM and IgG, respectively. These components were synthesized in spleen cells and were precipitated by the heat-inactivated *S. aureus* included in the immune reaction to facilitate the precipitation of immune complexes.

Two-dimensional gel electrophoresis (Fig. 1*A*) showed that both the 47,000- and the 46,000-M_r bands could be resolved into multiple components with discrete isoelectric points, ranging from about 5.85 to 6.5. This apparent heterogeneity of the H-2 antigens most likely represents intracellular modifications involving extensive glycosylation, which has been postulated to be important for the regulation of their function (22). β_2 microglobulin was also resolved into two spots, a major component with an isoelectric point of 6.7 and a minor component at 6.55.

Cell-Free Translation of mRNAs Coding for H-2 Antigens. Poly(A)-containing RNA was obtained by fractionation of total RNA from livers of A/J mice on an oligo(dT)-cellulose column. The bound fraction was eluted and translated in a rabbit reticulocyte lysate made largely mRNA-dependent by treatment with micrococcal nuclease.

Analysis by polyacrylamide/NaDodSO$_4$ gel electrophoresis of immunoprecipitates from [^{35}S]methionine-labeled cell-free translation products (Fig. 1*B*), using either normal rabbit serum (lane 1) or anti-H-2^a serum (lane 2), showed two components migrating as a doublet with apparent M_rs of 45,000 and 44,500 that were specific to the immune serum. They may represent the products of the two mRNA species, one specific to the *H-2K* locus and the other specific to the *H-2D* locus. Both proteins

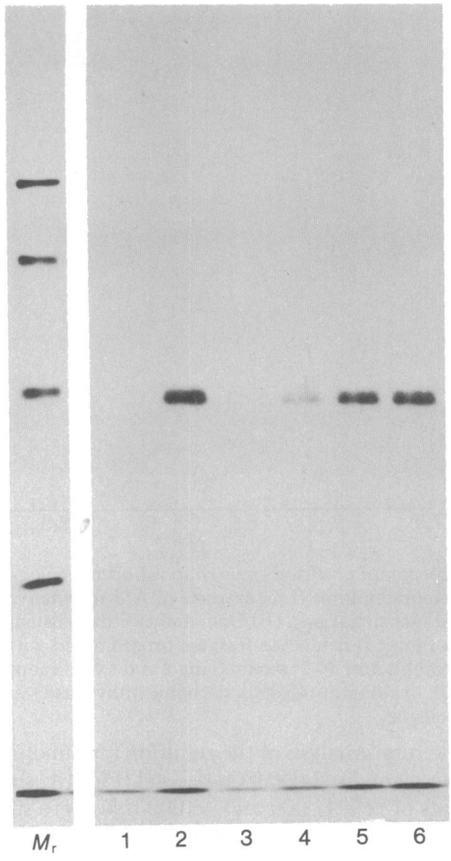

FIG. 2. Immunoprecipitation of cell-free translation products with anti-H-2^a in the presence of purified H-2^a antigens. [^{35}S]Methionine-labeled cell-free translation products, synthesized in rabbit reticulocyte lysate programmed with poly(A)-containing RNA from livers of A/J mice, were immunoprecipitated with either normal rabbit serum (lane 1) or rabbit anti-H-2^a serum (lane 2) in the presence of highly purified H-2^a antigens at 10 μg (lane 3), 1 μg (lane 4), and 0.1 μg (lane 5), and bovine serum albumin at 10 μg (lane 6). The M_r markers used were the same as those in Fig. 1.

migrated in a two-dimensional gel as single components with an isoelectric point of 5.9 (Fig. 1*B*). A component equivalent to β_2 microglobulin was also synthesized and precipitated by the anti-H-2^a serum. This cell-free translation product, migrating at the bottom of the gel, had an isoelectric point similar to that of β_2 microglobulin precipitated from spleen cells.

Characterization of H-2 Products Synthesized *In Vitro*. The immunoprecipitation of the cell-free products by the rabbit anti-H-2^a serum could be inhibited by the addition of purified H-2^a antigen (Fig. 2). The extent of inhibition was proportional to the amount of purified H-2^a added. Whereas the presence of 10 μg of H-2^a resulted in complete inhibition of immunoprecipitation (lane 3), the presence of an equivalent amount of bovine serum albumin had no detectable effect (lane 6). This strongly suggests that the cell-free products immunoprecipitated by the anti-H-2^a serum are antigenically related to the H-2^a antigens.

Further verification of the structural identity of the cell-free translation products and the purified H-2^a antigens was established by two-dimensional tryptic peptide mapping. The peptides produced by trypsin digestion of purified ^{14}C-labeled *p*H-2^a antigens and of [^{35}S]methionine-labeled cell-free products were resolved on thin-layer cellulose plates by electrophoresis and chromatography (Fig. 3). Approximately 30 of the expected 31 tryptic peptides of the purified-H-2^a (13), labeled

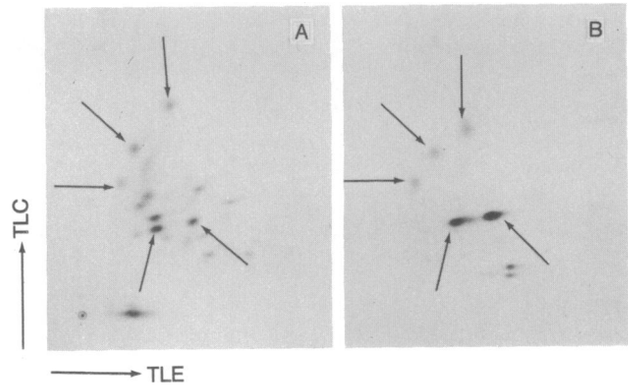

FIG. 3. Two-dimensional tryptic peptide mapping of purified H-2^a and cell-free synthesized products. (*A*) H-2^a purified from livers of A/J mice was digested with trypsin, and the tryptic digest was labeled with ethyl[1-^{14}C]acetimidate. (*B*) [^{35}S]Methionine-labeled cell-free products, synthesized in the rabbit reticulocyte lysate programmed with poly(A)-containing RNA obtained from livers of A/J mice, were precipitated with anti-H-2^a and the immunoprecipitates were fractionated on a 10% polyacrylamide/NaDodSO$_4$ gel. The 45,000- and 44,500-M_r components were detected by autoradiography, eluted together, and digested with trypsin. The tryptic digests were analyzed on thin-layer cellulose plates by electrophoresis (TLE), followed by chromatography (TLC). The arrows indicate common peptides.

with ethyl[1-^{14}C]acetimidate, were resolved (Fig. 3*A*). Labeling of amino groups by this procedure is not expected to affect the mobility of the resulting tryptic peptides (21). The two-dimensional map of the cell-free synthesized H-2 antigens showed five major [^{35}S]methionine-containing peptides (Fig. 3*B*), which agrees well with the methionine content of H-2 obtained by amino acid analysis (23). All five of these peptides corresponded in mobility to ^{14}C-labeled peptides obtained from purified H-2^a antigens. Although these corresponding peptides from purified H-2^a antigens have not yet been shown to contain methionine, the resolution of the majority of the expected peptides in the two-dimensional map favors this interpretation. Attempts to obtain tryptic maps of [^{35}S]methionine-labeled H-2, immunoprecipitated from splenic lymphocytes, were not successful.

Partial Purification of mRNAs for the H-2 Antigens. In an attempt to enrich for the mRNAs encoding the H-2 antigens, poly(A)-containing RNA was prepared separately from free polysomes and membrane-bound polysomes (Fig. 4 *Left*). Both RNA fractions were found to be translated equally efficiently in the rabbit reticulocyte lysate. However, although the extent of incorporation of [^{35}S]methionine per unit of poly(A)-containing polysomal RNA added was identical for the two RNA preparations, the amount of H-2 synthesized per unit was 3-fold higher for membrane-bound polysomal RNA (lane 4) than for free polysomal RNA (lane 2). Because the total yield of poly(A)-containing RNA from membrane-bound polysomes was greater than from free polysomes, it is concluded that up to 95% of the H-2 mRNAs were associated with membrane-bound polysomes.

The membrane-bound polysomes were further sedimented on a sucrose gradient in order to estimate the size of the H-2^a-synthesizing polysomes (Fig. 4 *Right*). The large amount of polysomes and relatively small amount of monosomes detected are indicative of minimal degradation by nucleases and minimal run-off of polysomes during tissue and sample preparations. The histogram shows the relative amount of H-2 synthesized when poly(A)-containing RNA from fractions across the sucrose gradient were assayed in the reticulocyte cell-free system. A

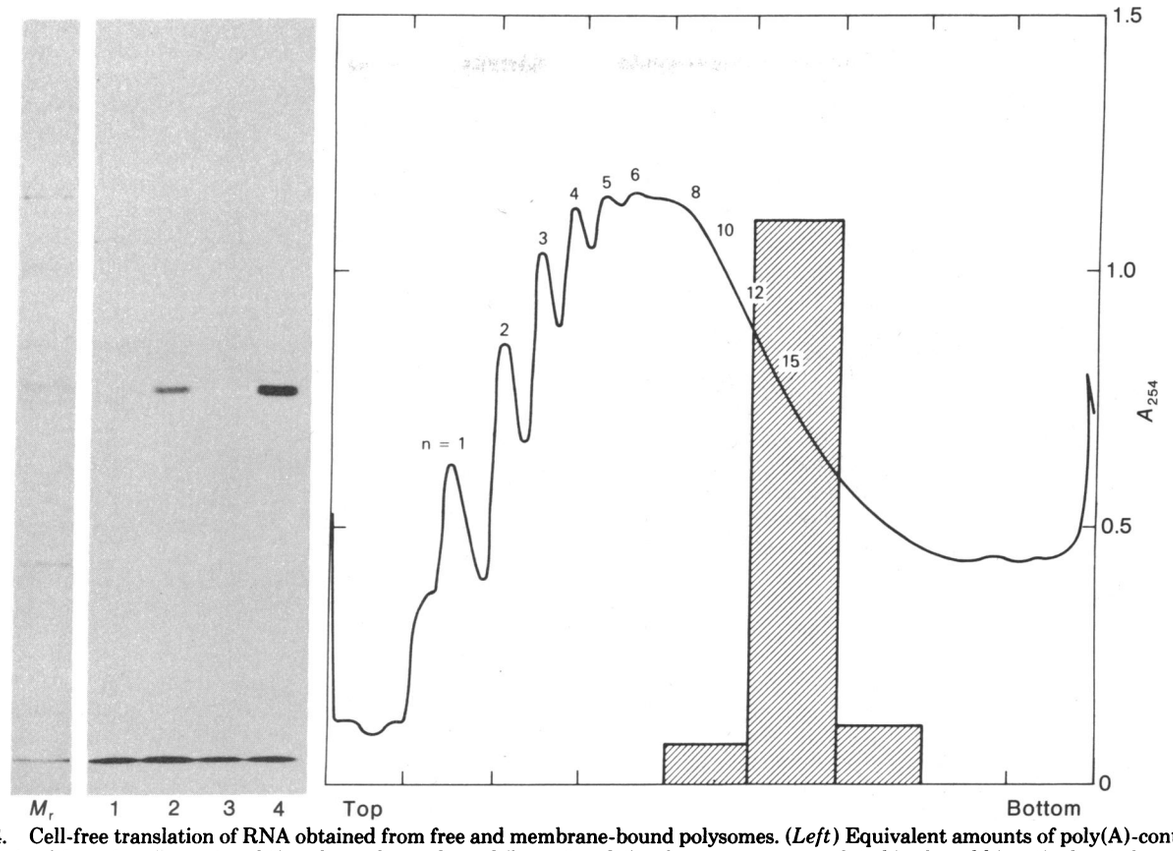

FIG. 4. Cell-free translation of RNA obtained from free and membrane-bound polysomes. (*Left*) Equivalent amounts of poly(A)-containing RNA obtained from free (lanes 1 and 2) and membrane-bound (lanes 3 and 4) polysomes were translated in the rabbit reticulocyte lysate. The cell-free translation products were immunoprecipitated with either normal rabbit serum (lanes 1 and 3) or rabbit anti-H-2^a serum (lanes 2 and 4). M_r markers used were the same as those in Fig. 1. (*Right*) Membrane-bound polysomes were fractionated on a 15–40% sucrose gradient. Poly(A)-containing RNA from each fraction was obtained and translated in the rabbit reticulocyte lysate. The relative amount of the 45,000- and 44,500-M_r components immunoprecipitated by the anti-H-2^a serum from each fraction (histogram) was estimated by densitometric tracings of autoradiograms.

discrete size-class polysome, representing about 16 ribosomes per mRNA molecule, was found to be synthesizing H-2 polypeptides. This suggests that the mRNAs for the H-2 antigens are actively being translated.

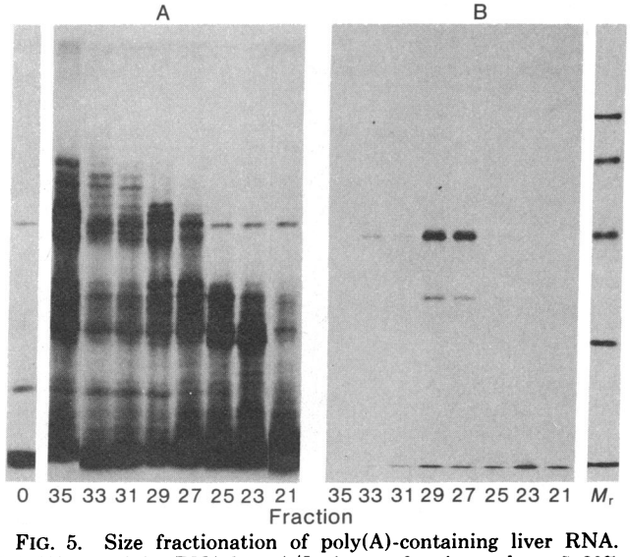

FIG. 5. Size fractionation of poly(A)-containing liver RNA. Poly(A)-containing RNA from A/J mice was fractionated on a 5–20% sucrose gradient, and each fraction was translated in the rabbit reticulocyte lysate. (*A*) Total cell-free translation products. Fraction 0 indicates the endogenous activity of the lysate with no added poly(A)-containing RNA. (*B*) Immunoprecipitates of the cell-free translation products obtained with the rabbit anti-H-2^a serum. M_r markers used were the same as those in Fig. 1.

To determine the size of the H-2 mRNAs, total liver poly(A)-containing RNA was fractionated by centrifugation on a 5–20% sucrose gradient (Fig. 5). An aliquot of each fraction was translated *in vitro*, and the cell-free translation products were immunoprecipitated with the anti-H-2^a serum. Fractions from a selected region of the gradient are shown in Fig. 5, either before (*A*) or after (*B*) immunoprecipitation with anti-H-2^a serum. A major portion of the mRNA encoding the H-2 antigens was found to sediment at about 17 S (fractions 27–29), relative to 28S and 18S ribosomal RNA fractionated on a parallel sucrose gradient. The mRNA for β_2 microglobulin was found to sediment at about 10 S (fraction 23) on the same sucrose gradient.

DISCUSSION

Because it is believed that H-2 antigens play a number of key roles in the immune response to antigens and in allograft rejection, there is currently much interest in the study of the organization and regulation of these genes. As a first step in the study of this complex system, we have identified and partially characterized the mRNAs coding for the H-2K and H-2D antigens.

The H-2 mRNAs sediment at about 17 S and are found predominantly associated with membrane-bound polysomes. They are apparently actively being translated in the liver, where as many as 16 ribosomes are found associated with each molecule of H-2 mRNA.

The H-2 antigens synthesized in the rabbit reticulocyte cell-free system were identified by immunoprecipitation and

tryptic peptide mapping. They have apparent M_rs of 44,500 and 45,000. Because these antigens were synthesized under conditions in which there was no glycosylation of nascent chains, they are expected to have lower apparent M_rs than their *in vivo* counterparts, which are glycoproteins of 46,000 and 47,000 M_r. The carbohydrate moieties present in these H-2 antigens have been estimated to contribute about 3000 M_r (5); the cell-free products we detected, therefore, are slightly larger than expected. The most likely explanation is that the cell-free products represent precursor molecules containing extra amino acid sequences that are not present in the glycosylated forms. Signal sequences of about 3000 M_r have been found on precursors of secretory and membrane proteins (24, 25). Indeed, in the case of the HLA antigens, the cell-free translation products have been shown to be larger than the nonglycosylated forms of these antigens (26).

The identification and purification of these H-2 mRNAs are crucial for the molecular cloning of the *H-2* genes. The availability of cloned *H-2* sequences will undoubtedly lead to a better understanding of the organization and regulation of this highly polymorphic family of genes.

U.F. is a Visiting Scientist from Regina Elena Cancer Institute, Rome, Italy.

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