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Liver Antigens Detected by Liver Antinucleolar Antibodies[1] (40416)

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Previous studies from this laboratory using the immunodiffusion technique showed that normal liver nuclear and nucleolar chromatin contained several antigens which were not found in similar extracts of Novikoff hepatoma (1–3).

Following the work of Abelev *et al.* (4) a number of reports have shown the presence of specific liver antigens. Recently a nuclear protein was found in the chromatin of normal liver cells but not in hepatoma cells (5); this protein had a MW of 20,000 and was reported to be related to a mouse urinary protein.

Many studies have indicated that tumors contain antigens different from their cells of origin (3, 6). Some are known to be tumor specific transplantation antigens and others oncoembryonic antigens; their presence indicates alterations in the gene readouts to include readouts of fetal genes (7). Conversely, many proteins present in normal tissue are not found in tumor cells (7–9). Gene repressors may be absent from proliferating tumor cells which may then exhibit unrestrained growth (7).

The present study was designed to investigate the relationships of the antigens previously found in liver nucleoli (2) to antigens in other cellular fractions of liver and to test a broader range of tissues for the antigen. As part of this study, liver antigens were purified on an antibody affinity column. Interestingly, GC-rich RNA of approximately 200,000 MW was found in the purified antigen.

Materials and methods. Liver nucleolar antibody. As previously reported (10) rabbits were immunized with Ca^{2+} sucrose nucleoli isolated from normal rat liver. The antiserum was precipitated with 50% saturated ammonium sulfate to obtain a crude immunoglobulin fraction (11). The antibody preparation contained approximately 30 mg of protein/ml.

Preparation of antigens. Several antigens were prepared as previously described (2, 3); extracts were made of liver or tumor nuclei and nucleoli with 0.075 *M* NaCl/0.025 *M* EDTA followed by 0.01 *M* Tris–HCl (pH 8). Chromatin was extracted with 0.6 *M* NaCl/0.01 *M* Tris–HCl (pH 8). The solutions contained 1 m*M* PMSF (phenylmethylsulfonylfluoride). Each extract was centrifuged at 100,000*g* for 18 hr and the supernatant was concentrated on Amicon UM-10 membranes.

Saline soluble tissue extracts were prepared from normal liver, regenerating liver, kidney and spleen. The tissues were passed through a Harvard press and suspended in 0.15 *M* NaCl. The pressate was centrifuged at 1000*g* for 4–5 min and then resuspended in saline for two additional washes; the first supernatant is referred to as w1 and the next two are referred to as w2 and w3. The final washed pellet was suspended in 5 vol of saline and extracted at 4° or 37° for 1 hr. The extracted pellet was centrifuged at 2000*g* for 30 min and the resultant supernatant contained the saline soluble tissue antigens (L, K or S-liver, kidney or spleen, respectively).

Liver and tumor polysomes were prepared as previously described (12); liver or tumor cells were homogenized in TKM (0.06 *M* Tris–HCl/0.05 *M* KCl/0.005 *M* $MgCl_2$/pH 7.4). The postmitochondrial supernatant was treated with 0.5% sodium deoxycholate and underlayered with 1.0 *M* sucrose and 2.0 *M* sucrose layers (in TKM). The tubes were centrifuged for 17 hr at 100,000*g*. The resultant supernatant as well as the 1.0 *M* and 2.0 *M* sucrose layers from the liver preparation were used as antigens. The tumor and liver polysomal pellets were extracted with 0.15 *M* NaCl for 1 hr at 4° or 37°. Following centrifugation at 2000*g* for 30 min, the polysomal supernatants were also used as antigens.

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Ouchterlony immunodiffusion plates and antigen units. The immunodiffusion plates were prepared from 0.8% agarose dissolved in 0.15 *M* NaCl/0.01 *M* sodium phosphate/0.5% sodium azide (pH 7.2). Each globulin well contained 25 μl (30 mg/ml) and each antigen well contained 25 μl of the specific antigen (5–15 mg/ml). The precipitin lines were allowed to develop at room temperature in a moist chamber for 48 hr (13). A final observation was made after 7 days.

Antigen units (A.U.) were determined by dilution of the various antigens in the presence of constant antibody. The amount of antigen in the dilution below which no distinct precipitin line was observed was defined as 1 A.U. The antigens were diluted with 0.0175 *M* sodium phosphate/0.15 *M* NaCl (pH 6.3); the dilution was 1:5–1:40.

Affinity chromatography. Liver antinucleolar antibodies (30 mg protein/ml in 0.01 *M* sodium phosphate/0.15 *M* NaCl, pH 7.2) were bound to CNBr-Sepharose (14). The bound antibody was mixed with liver antigen extract in 0.15 *M* NaCl by end-over-end rotation for 18 hr at 4°. Unbound and loosely bound proteins were removed by washing the column with 0.0175 *M* sodium phosphate/1.0 *M* NaCl (pH 6.3). Elution was carried out with 0.2 *M* Tris–HCl/0.5 *M* NaCl with or without 0.2 *M* putrescine (pH 11). The eluted material was monitored for absorbance at 260 and 280 nm. The eluate was dialyzed extensively against 0.0175 *M* Na phosphate/0.15 *M* NaCl (pH 6.3). The dialyzed sample was concentrated in an Amicon apparatus (UM-10 membrane).

Ribonucleic acid analysis. RNA was extracted from the eluted antigen by the sodium dodecyl sulfate/phenol procedure (15). The extracted RNA was analyzed on an 8% polyacrylamide gel. The nucleotide composition was determined according to the method of Randerath and Randerath (16).

Absorption of the antisera with nucleoli. As previously reported (2), the liver nucleolar antibodies were absorbed with approximately 10 mg/ml of tumor nucleoli by incubation for 1 hr at 37° and for an additional hour at room temperature. The treated globulin was centrifuged at 1800*g* for 30 min to pellet the nucleoli. The supernatant globulin was removed and used as absorbed liver nucleolar antibody.

Results. Liver soluble antigens. Three antigens were detected in liver nucleoli and 0.15 *M* NaCl extracts of whole liver by antibodies to liver nucleoli (Fig. 1); antigens Ln-1 (arrow) and Ln-2 (arrowhead) in nucleolar extracts (Ln) formed dense immunoprecipitin bands with the antinucleolar antibodies (Ab). Antigen Ln-1 in liver extracts (L) formed an equally dense immunoprecipitin band with the antinucleolar antibodies and a third antigen, Ln-3 (double arrowhead), was detected in the whole liver 1000*g* supernatants of three successive 0.15 *M* NaCl liver extracts (w1–w3) as noted in Figs. 1 and 2C. The identity of antigen Ln-1 in whole liver (L) and liver nucleoli (Ln) is indicated in Fig. 2A.

Tissue distribution. Antigen Ln-1 was not found in Novikoff hepatoma, nuclear (TNC) and nucleolar (TnC) chromatin extracts (Fig. 2B) or in Novikoff hepatoma polysomal extracts (TP, Fig. 2A); all the tumor preparations contained antigen Ln-2 (Fig. 2A and 2B). Table I shows that regenerating liver contained the same antigens Ln-1 and Ln-3 as normal liver (Fig. 2D). However, like the Novikoff hepatoma, spleen (S) contained

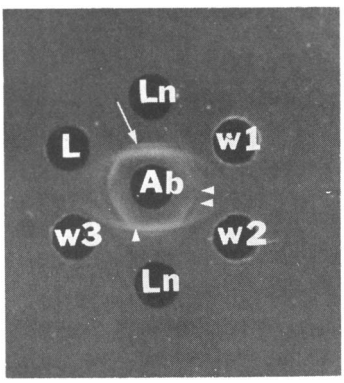

FIG. 1. Immunodiffusion plate with Ig from antiserum to liver nucleoli in the center well. The following antigens were in the outer wells: (Ln) the 0.15 M NaCl extract of liver nucleoli; the supernatants from the 3 consecutive 0.15 *M* NaCl extracts of liver pressate designated (w1), (w2) and (w3); the 0.15 *M* NaCl extract (1 hr) of the "washed" liver pressate (L). The following immunoprecipitin bands formed between the nucleolar antibodies (Ab) and the antigens were: Ln-1 (arrow) with antigens w1, w2, w3, L and Ln; Ln-2 (arrowhead) with antigen Ln; and Ln-3 (double arrowheads) with antigens w1, w2, w3 and L. Extraction of liver pressate with 0.15 *M* NaCl readily solubilized antigens Ln-1 and Ln-3.

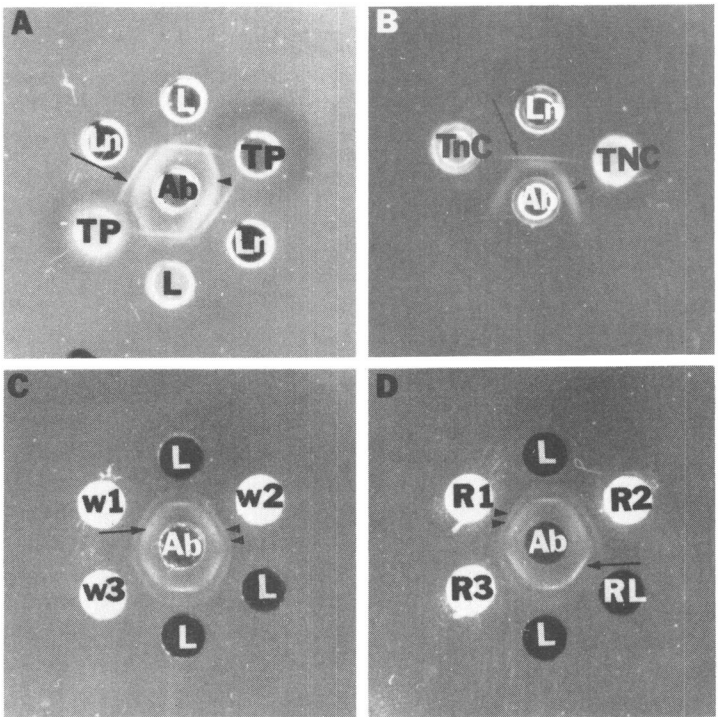

FIG. 2A. Immunodiffusion plate with antibodies to liver nucleoli (Ab) in the center well. The antibodies formed immunoprecipitin bands with antigens Ln-1 (arrow) and Ln-2 (arrowhead) in the 0.15 *M* NaCl extract of liver nucleoli (Ln) and antigen Ln-2 (arrowhead) in the 0.15 *M* NaCl extract of tumor polysomes (TP). B. Immunodiffusion plate with antibodies to liver nucleoli in the center well (Ab). The antibodies formed immunoprecipitin bands with antigen Ln-2 (arrowhead) in tumor nuclear (TNC) and nucleolar chromatin (TnC), and antigens Ln-1 (arrow) and Ln-2 (arrowhead) in liver nucleoli (Ln). C. Immunodiffusion plate which contains antibodies to liver nucleoli in the center well. The antibodies formed precipitin bands Ln-1 (arrow) and Ln-3 (double arrowhead) with the antigens from the 0.15 *M* NaCl supernatants of liver (w1, w2, w3 and L). D. Immunodiffusion plate with antibodies to liver nucleoli (Ab) in the center well. The antibodies formed precipitin bands with the 0.15 *M* NaCl supernatants of regenerating liver (R1, R2, R3 and RL). Well (L) contains the 0.15 *M* NaCl extract of normal liver. Precipitin bands formed with antigens Ln-1 (arrow) and Ln-3 (double-arrowhead).

only antigen Ln-2 (arrowhead, Fig. 3A). When liver antinucleolar antibodies were preabsorbed with tumor nucleoli, the Ln-2 band of the spleen did not form (Fig. 3B). The immunoprecipitin band for the kidney was relatively weak and overlapped the Ln-1 band (Fig. 3A). With antibody preabsorbed by tumor nucleoli (Fig. 3B), the Ln-1 band formed with liver antigen (L) was very dense but the kidney band was very faint (large arrowhead, Fig. 3B).

Antigen Ln-1 is apparently not a serum protein such as albumin. The precipitin bands (Fig. 4) formed between liver nucleolar antibodies (Ab) and liver antigen (L - arrow) and rat albumin antibodies (AAb) and albumin antigens (A-double arrow) are different. Liver nucleolar antibodies did not form bands with normal rat serum.

Distribution of liver antigen Ln-1. Table II shows the quantity of antigen units (A.U.) of antigen Ln-1 in various liver fractions. Extraction of liver nuclei and nucleoli with 0.075 *M* NaCl/0.025 *M* EDTA (pH 8) released approximately 90% of antigen Ln-1 from nuclei and nucleoli. The 0.01 *M* Tris–HCl extract contained very little additional liver antigen but more was extracted with 0.6 *M* NaCl/0.01 *M* Tris–HCl (pH 8). In discontinuous sucrose gradients of the cytosol fraction, the supernatant and the 1.0 *M* sucrose fractions contained 132 A.U. and 87 A.U./g of liver (Table II). The 2.0 *M* sucrose layer had only 25 A.U./gram of liver and none was

detected in the 0.15 *M* NaCl extract of the liver polysomal pellet. In addition, Table II shows the total antigen units (A.U.) for antigen Ln-1 in the various fractions. The antigen units (A.U.) per mg of protein ranged between 100 and 200 for the three fractions studied, namely cytosol, nuclei and nucleoli. These data indicate the cytosol contains 99%, the nuclei 1% and the nucleoli 0.03% of the total antigen.

Affinity chromatography purification of Ln-1 antigen. The elution profile of the liver antigen from an affinity column prepared with antibodies to liver nucleoli is shown in Fig. 5. The bulk of the proteins did not bind to the affinity column containing the nucleolar antibodies (flow-through fraction). The flow-through fraction was collected batchwise and was found to contain 98.4% of the total protein by Lowry analysis (17). The bound fraction was eluted with 0.2 *M* Tris–HCl/0.5 *M* NaCl (pH 11) at the point indicated by an arrow. The bound fraction contained approximately 1.6% of the total proteins that were added to the antibody column (17). The absorbance of the eluted fraction was consistently higher at 260 nm than at 280 nm even after dialysis and concentration; i.e., the ratio of the absorbance of 260 nm and 280 nm was 1.7. Figure 6 shows that the eluted antigen (BL-liver bound) exhibited antigenic identity to the crude liver antigen (L).

TABLE I. DISTRIBUTION OF LIVER ANTIGENS IN VARIOUS TISSUES.[a]

Tissue	Antigen		
	Ln-1	Ln-2	Ln-3
Liver extracts	+	+	+
Liver nucleoli	+	+	tr
Regenerating liver	+	+	+
Novikoff hepatoma	−	+	−
Spleen	−	+	−
Kidney*	−	−	−
Rat serum	−	−	−

[a] The three 0.15 *M* NaCl soluble antigens Ln-1, Ln-2 and Ln-3 are listed as present (+), trace (tr) or absent from specific tissues (−).

* In the kidney, an antigen was identified that formed a band that overlapped with the Ln-1 band but did not exhibit identity with Ln-1.

Ribonucleic acid analysis. The polyacrylamide gel of the phenol-SDS extract of the eluted antigen is shown in Fig. 7 which shows three bands (left) were present. The gel on the right contains marker RNAs. The approximate MW of the bands were 200,000 as determined by comparative migration with 18S rRNA and lower MW markers. The nucleotide composition of the RNA was determined by the Randeraths' procedure (16): AMP 14, UMP 16, GMP 34, CMP 36. The GMP + CMP/AMP + UMP ratio was 2.33.

Discussion. The present results show that the rabbit antibodies to normal rat liver nucleoli detect several antigens in normal rat liver and other tissues. Antigens Ln-1 and Ln-3 which were present in rat liver nucleoli

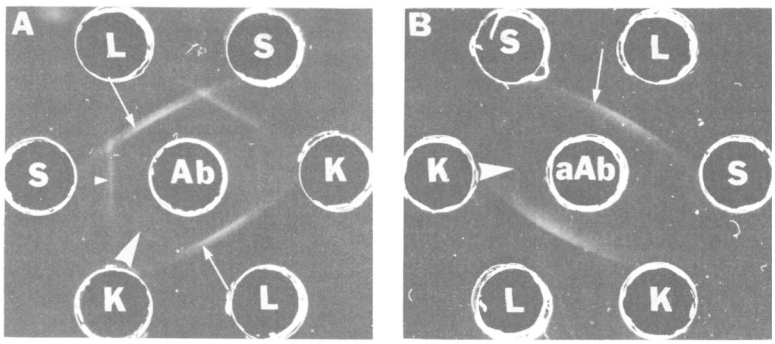

FIG. 3A. Immunodiffusion plate with antibodies to liver nucleoli (Ab) in the center well. The antibodies formed a precipitin band (large arrowhead) with the 0.15 *M* NaCl kidney extract (K) and with antigen Ln-1 (arrow) from liver (L). The antibodies also formed an immunoprecipitin band with antigen Ln-2 (arrowhead) in the 0.15 *M* NaCl extract of spleen (S). B. Immunodiffusion plate with liver nucleolar antibodies preabsorbed with tumor nucleoli (aAb) in the center well. The preabsorbed antibodies formed immunoprecipitin bands with antigen Ln-1 (arrow) in the 0.15 *M* NaCl extract of liver (L). A faint immunoprecipitin band (large arrowhead) formed with the 0.15 *M* NaCl extract of kidney (K). The immunoprecipitin band with antigen Ln-2 of spleen did not form with the preabsorbed antiserum.

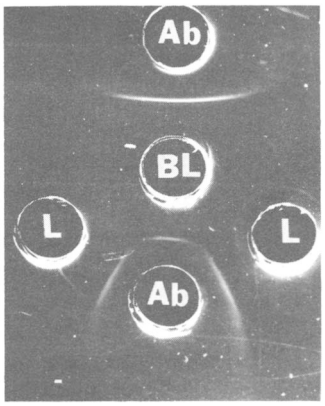

FIG. 4. Immunodiffusion plate which contains antibodies to liver nucleoli (Ab) in the upper well. The liver nucleolar antibodies formed immunoprecipitin bands (arrow) with the liver antigen (L). Immunoprecipitin bands (double arrows) formed between antibody to albumin (AAb) and the albumin antigen (A). There was no reaction between albumin antibody (AAb) and liver antigen (L) or liver nucleolar antibodies (Ab) and albumin antigen (A).

were also found in 0.15 *M* NaCl extracts of pressates of normal and regenerating liver but not in other tissues. Antigen Ln-2 was detected in Novikoff hepatoma, liver and spleen. Absorption of the antibodies with Novikoff hepatoma nucleoli prevented formation of the Ln-2 immunoprecipitin band in spleen and other tissues. None of the antigens was detected in rat serum. In kidney, another antigen was detected that overlapped but was not identical to antigen Ln-1.

Although liver specific antigens were initially found in nucleoli in this laboratory (2, 3), the amounts in nuclei and nucleoli are very small by comparison with the cytoplasm. The presence of the antigens in the nucleolar and nuclear preparations may be significant in gene function inasmuch as small amounts of the antigen(s) were consistently found in the chromatin prepared from nuclei and nucleoli (Table II). Inasmuch as not all the antigen(s) was extracted with 0.15 *M* NaCl but was sufficiently tightly bound to the chromatin that 0.6 *M* NaCl was required for its extraction, there may be special chromatin binding sites for these antigens. To determine possible functional roles of the antigens Ln-1, Ln-2 and Ln-3, studies are now being initiated on the effects of these antigens on isolated liver nuclear and nucleolar preparations.

TABLE II. ANTIGENIC ACTIVITY OF LIVER FRACTIONS.[a]

Fractions	*A. U./mg protein*	*A. U./g liver*	*% of Total/ g liver*
Cytosol			
Supernatant	55	132	54
Sucrose layer 1.0 *M*	82	87	36
Sucrose layer 2.0 *M*	34	25	10
Polysomal pellet	0	0	0
Nuclear			
NaCl–EDTA	178	2.4	91
0.01 *M* Tris-HCl	4.8	0.03	1
0.6 *M* NaCl	15.5	0.21	8
Nucleolar			
NaCl–EDTA	101	0.07	90
0.01 *M* Tris–HCl	3.4	0.003	4
0.6 *M* NaCl	5.8	0.005	6
Totals			
Cytosol	171	244	99.0
Nuclei	198	2.6	1.0
Nucleoli	110	0.08	0.03

[a] The relative amounts of antigen Ln-1 were determined for the different fractions of liver cytosol in a discontinuous TKM sucrose gradient (Materials and Methods), for sucrose Ca^{2+} liver nuclei and nucleoli and for the totals of each fraction. The antigenic unit, A.U., was defined as the amount of antigen in the dilution below which no distinct precipitin band was observed. The results show antigen units/mg protein and antigen units/gram of liver. The percent of the total is also shown.

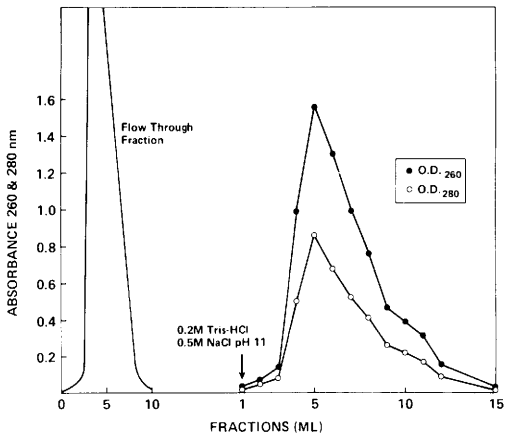

FIG. 5. The 0.15 *M* NaCl extract of liver was bound to a Sepharose affinity column which was covalently linked to liver antinucleolar antibodies. The bound antigens were eluted with 0.2 *M* Tris-HCl/0.5 *M* NaCl (pH 11.0). The elution profile shows the absorbance at 260 and 280 nm. By Lowry (17) analysis, the flow-through fraction contained approximately 98.4% and the antigen peak contained 1.6% of the total protein.

The localization of the antigen in the cytoplasm was of interest. Following ultracentrifugation in a discontinuous sucrose gradient, the antigen was mainly in the supernatant and in the 1.0 *M* sucrose layers (Table II). The presence of RNA (M.W. approximately 200,000) suggests that the antigen Ln-1 may be a ribonucleoprotein. The associated RNA is GC-rich, i.e. the GMP + CMP/AMP + UMP ratio is 2.33. In preliminary studies, the three RNA bands were found (18, 19) to have similar compositions (band 1, fastest AMP, 13%, UMP 16%, GMP 32%, CMP 39%; band 2, AMP 11%, UMP 17%, GMP 36%, CMP 36%; band 3, AMP 15%, UMP 18%, GMP 33%, CMP 34%) which suggests they may be conformers of one RNA species but more detailed analysis is required to establish this possibility. Recently, Miller *et al.* found three similar RNA bands (20). It is of interest that Nakamura *et al.* (21) found similar RNA bands in nucleoli. However, more detailed studies are needed to determine whether there is chemical identity of these RNA species.

Summary. Three antigens (Ln-1–Ln-3) in extracts of normal rat liver were detected by immunodiffusion analysis using rabbit antibodies to liver nucleoli. In analyses of a series of tissues, antigens Ln-1 and Ln-3 were found only in normal and regenerating liver. Antigen Ln-2 was found in several tissues studied but not in kidney or rat serum; in kidney, an antigen was found that formed an overlapping band with antigen Ln-1 but did not exhibit identity with antigen Ln-1. In liver, approximately 99% of the antigen Ln-1 was in the cytosol and 1% was in the nuclei. In the extracts of nuclei and nucleoli, approximately 6–8% of this antigen was in the chromatin fraction from which it was extractable with 0.6 *M* NaCl. The antigens were purified on affinity columns which contained covalently bound liver antinucleolar antibodies. Interestingly, the bound antigen contained

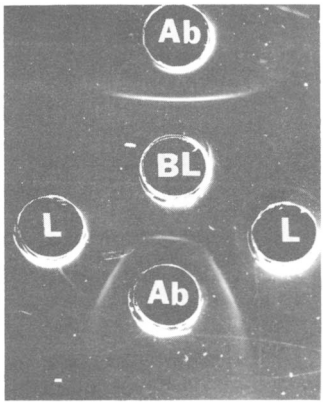

FIG. 6. An immunodiffusion plate which contains liver antinucleolar antibodies (Ab) in the top and bottom wells. The bound fraction (BL) formed an identity band with the liver antigen (L).

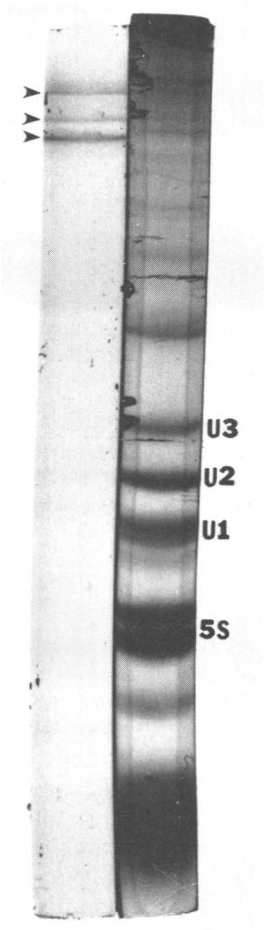

FIG. 7. The 8% agarose gel on the left contains three ribonucleic acid bands (arrows). The RNA was extracted from the eluted antigen by the phenol-SDS method and its nucleotide composition was analyzed by the Randerath procedure (16). The gel on the right contains RNA markers designated 5S, U1, U2 and U3. The molecular weights are, respectively: 40,000, 57,000, 65,000 and 71,000 daltons (18).

GC-rich RNA of approximately 200,000 MW.

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