Human Cytomegalovirus RNAs Immunoprecipitated by Multiple Systemic Lupus Erythematosus Antisera

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SUMMARY

The association of human cytomegalovirus (HCMV) RNAs with ribonucleoprotein particles that react with antibodies from patients with systemic lupus erythematosus was tested by immunoprecipitation with multiple patients' sera. A major late 2.8 kb RNA and several minor RNAs encoded by the HCMV long repeat region were immunoprecipitated from HCMV-infected cells by La, Ro and, much less abundantly, Sm autoimmune antisera. The exact location of these RNAs was determined by high resolution R-loop mapping and found to be between 0.8093 and 0.8189 map units. The 2.8 kb RNA is polyadenylated and associated with polysomes but does not appear to be spliced. Immunoprecipitation was not seen using normal or other autoimmune antisera. In addition, immunoprecipitation was specific to these RNAs in that other abundant HCMV RNAs were not immunoprecipitated. It was also found that the addition of increasing amounts of purified La antigen to infected cell lysates inhibited immunoprecipitation of the 2.8 kb RNA by La antiserum. The data suggest that specific HCMV RNAs may interact with cellular ribonucleoproteins known to be involved in post-transcriptional regulation of gene expression.

INTRODUCTION

Various small nuclear and cytoplasmic RNAs of eukaryotic cells form ribonucleoprotein (RNP) complexes that appear to be important at one or more steps in the process of gene expression (reviewed in Steitz et al., 1982). Antisera from patients with autoimmune diseases, such as systemic lupus erythematosus (SLE), have allowed identification of separate RNP classes by recognition of antigenic protein moieties unique to each class. Four of the best characterized RNP classes are La, Ro, (U1)RNP and Sm. La RNPs contain a 45K La antigen bound to the 3'-terminal polyuridylate residues of RNA polymerase III transcripts (e.g. tRNA and SS rRNA precursors, adenovirus VA RNAs, and Epstein–Barr virus EBER RNAs) and of certain RNAs transcribed by viral RNA polymerases (e.g. vesicular stomatitis virus and rabies virus leader RNAs) (Kurilla et al., 1984; Kurilla & Keene, 1983; Pizer et al., 1983; Stefano, 1984; Steitz et al., 1982; Wilusz et al., 1983). The Ro RNPs, a subset of La RNPs, contain both a 60K Ro antigen and the La antigen bound to several species-specific RNAs (Steitz et al., 1982; Wolin & Steitz, 1984). The Sm RNP class contains at least nine small nuclear RNAs (U1, U2, U4, U5, U6, U7, U8, U9 and U10) bound by at least six different proteins (Hinterberger et al., 1983; Pettersson et al., 1984; Reddy et al., 1985; Steitz et al., 1982). The U1 RNA, in addition to its association with some of the Sm proteins, is also complexed with three unique proteins which are the (U1)RNP-specific antigens (Hinterberger et al., 1983; Steitz et al., 1982).

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Several key functions have been proposed for these RNPs. (U1)RNP and Sm RNPs have been implicated in mRNA splicing as well as polyadenylation. For example, U1, U2 and U5 RNPs bind to the 5' splice site, the intron region, and the 3' splice site, respectively. (U1)RNP and Sm antisera have been shown to inhibit both pre-mRNA splicing and polyadenylation (Black et al., 1985; Chabot et al., 1985; Mount et al., 1983; Padgett et al., 1983). La RNPs may be involved in the regulation of mRNA polyadenylation (Moore & Sharp, 1984) and/or protein translation. It has been shown that the adenovirus VA1 RNA which is precipitated by anti-La serum is indispensable for virus growth, apparently being required to maintain the activity of translation initiation factor eIF-2 in the infected cell (O'Malley et al., 1986; Thimmappaya et al., 1982). In addition, (U1)RNP, Sm and La antisera were found to immunoprecipitate both cellular and viral pre-mRNAs and mature spliced mRNAs, indicative of a role for these RNPs in mRNA processing (Hamelin et al., 1986). Although less is known about the role of Ro RNPs, it has been postulated that they may also be involved in regulation of mRNA translation (Wolin & Steitz, 1984).

We have found that specific human cytomegalovirus (HCMV) RNAs can be immunoprecipitated from cells by certain SLE antisera. The RNAs precipitated were found to be a distinct subset of the viral RNAs produced during infection and were mapped to the long repeat sequences. Of these, a 2.8 kb RNA was the most abundant, and was immunoprecipitated by La, Ro and Sm antisera, but not by (U1)RNP or other autoimmune antisera. Immunoprecipitation of the 2.8 kb RNA was inhibited by the addition of increasing amounts of La antigen. As this RNA is not spliced and has no known polypeptide products, its association with cellular SLE proteins and/or RNPs or both may be due to its involvement in the post-transcriptional regulation of cellular and viral gene expression or both in HCMV-infected cells.

**METHODS**

*Virus infection and [32P]orthophosphate labelling.* The human embryo lung fibroblast cell strain MRC-5 (ATCC, No. CCL 171) was cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% Nu-serum (Collaborative Research). The Towne strain of HCMV was a gift from R. LaFemina and G. Hayward (Johns Hopkins University, Baltimore, Md., U.S.A.). Viral infections were initiated at a multiplicity of infection of about 2 p.f.u./cell and after a 1 h adsorption period were maintained in DMEM containing 2% Nu-serum (Kilpatrick & Huang, 1977). Mock-infected and HCMV-infected cells were labelled with [32P]orthophosphate after 1 h adsorption by the addition of 100 μCi/ml [32P]orthophosphate in phosphate-free DMEM containing 2% dialysed foetal calf serum.

*Specificity of antisera.* Anti-DNA, anti-centromere and anti-nucleolus antisera were established reference antisera (Centers for Disease Control, Atlanta, Ga., U.S.A.). Some La, Ro, Sm and (U1)RNP antisera used in these experiments were a gift from Dr Henry Rothberger (Section of Rheumatology, Bowman Gray School of Medicine) and were characterized as being monospecific by immunofluorescence, counter-immunoelectrophoresis, and double immunodiffusion experiments. Other preparations of La, Ro, Sm and (U1)RNP antisera and Sc1-70 antisera (anti-DNA topoisomerase I; Shero et al., 1986) were obtained from Alpha Antigen.

*RNP immunoprecipitation.* RNP complexes were immunoprecipitated from cells essentially as described by Petterson et al. (1984). Briefly, cells were harvested at various times post-infection (p.i.), resuspended (1.5 × 10^8 cells/ml) in TBS (150 mM-NaCl, 50 mM-Tris-HCl pH 7.4) and, after gentle sonication, adjusted to 1 unit/ml RNAase (Calbiochem-Behring) in NET-2 buffer (150 mM-NaCl, 50 mM-Tris-HCl pH 7.4, 5 mM-EDTA, 0.05% NP40) followed by centrifugation at 12000 g for 5 min to remove any material which nonspecifically bound to Pansorbin cells (preclearing). A monospecific autoimmune antiserum (20 to 60 μl/1.5 × 10^7 cells) was added to the precleared sonicate which was then incubated at 2 °C for 15 min with gentle mixing. Pansorbin (10 μl Pansorbin/μl antisera) was then added and mixing continued for 15 min at 2 °C. The Pansorbin cells (with antibody-bound RNP complexes) were then pelleted, the supernatant was removed and saved for subsequent extraction of non-immunoprecipitated RNAs, and the immunoprecipitation pellet was washed five times with NET-2 at 4 °C. RNA was recovered from the pelleted immunoprecipitates and from supernatants by phenol and chloroform–isoamyl alcohol (24:1) extractions followed by ethanol precipitation in the presence of 250 μl/ml carrier yeast tRNA. After treatment with 1 mg/ml RNase-free DNase I (prepared as described by Maniatis et al., 1982) for 20 min at 37 °C, the RNA was then purified by organic extractions followed by ethanol precipitation as described above.
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Purification of polysome-associated RNA. Polysome-associated RNA was purified as described by Schreier & Stachelin (1973) with the following modifications. HCMV-infected cells were harvested, washed, resuspended in KMT (10 mM-KCl, 1.5 mM-magnesium acetate, 1 mM-dithiothreitol, 10 mM-Tris-HCl pH 7.6, 0.2 mM-EDTA), and disrupted by Dounce homogenization. After the nuclei had been removed (10 500 g for 10 min), the supernatant was adjusted to 1% deoxycholate, layered onto 1.0 and 1.8 M-sucrose step gradients and centrifuged at 110,000 g for 16 h. The polysomal pellets were resuspended in 1 vol. of TSB (0.01 M-Tris-HCl pH 7.0, 0.15 M-NaCl, 2 mM-MgCl2) and 2 vol. of TSES (0.01 M-Tris-HCl pH 7.9, 0.15 M-NaCl, 5 mM-EDTA, 0.2% SDS), extracted with phenol and chloroform-isooamyl alcohol and ethanol-precipitated.

**Northern and Southern hybridization.** RNAs were denatured by glyoxylation (Thomas, 1983), electrophoresed through vertical 1% agarose gels and electroblotted onto Nytran paper (Schleicher & Schuell) as described by the manufacturer. After the transfer, the filters were baked at 80 °C for 2 h and then boiled for 5 min in 20 mM-Tris-HCl pH 8.0 to remove glyoxal residues (Thomas, 1983). Prehybridization was conducted at 42 °C for 24 h in 5 × SSPE (1 × SSPE is 0.18 M-NaCl, 1 mM-EDTA, 10 mM-NaH2PO4-Na2HPO4 pH 7.7), 0.1% SDS, 5 × Denhardt’s solution [1 × contains 0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin (BSA)], 250 μg/ml denatured *Escherichia coli* DNA and 50% formamide. The prehybridization solution was replaced with fresh hybridization solution (same as prehybridization solution but with 2 × Denhardt’s) containing 1 μg denatured recombinant plasmid or HCMV DNA labelled with [α-32P]dCTP (1 × 107 to 5 × 107 c.p.m./μg) by nick translation as described by Maniatis et al. (1982). After incubation for 24 h at 42 °C, filters were washed twice for 15 min in 2 × SSC and 0.1% SDS at 50 °C, followed by two 15 min washes in 0.1 × SSC and 0.1% SDS at 75 °C (Thomas, 1983) and exposed for autoradiography using Kodak XAR-5 film and Cronex intensifying screens. For quantitative analysis, gels or blots were exposed to pre-flashed Kodak XAR-5 film and autoradiographs were scanned using a soft laser densitometer with digital integration (Biomed Instruments).

For Southern analysis, restriction enzyme-digested DNA was electrophoresed through vertical 0.7% agarose slab gels in E buffer (40 mM-Tris-HCl pH 7.2, 20 mM-sodium acetate, 1 mM-EDTA). The gels were then treated and the DNA electroblotted onto Nytran filters as described by the manufacturers (Schleicher & Schuell). Filters were incubated at 50 °C for 8 h in a modification of the prehybridization solution which contained 50% (v/v) formamide, 5 × Denhardt’s, 4 × SSC, 10 mM-NaH2PO4-Na2HPO4 pH 7.8, 10 mM-HEPES pH 7.8, 2 mM-EDTA and 500 μg/ml *E. coli* rRNA. Hybridization was conducted for 24 h at 50 °C in an identical but freshly mixed solution which contained 5 × 105 c.p.m. (approx. 1 μg) of 32P-labelled RNA probe. The filters were then washed five times for 2 min each in 2 × SSC, 10 mM-NaH2PO4-Na2HPO4 pH 7.4, 2 × Denhardt’s and 2 mM-EDTA at 60 °C, three times for 2 min in the same buffer containing 0.5% SDS at 60 °C, and twice for 2 min in 0.1 × SSC, 10 mM-NaH2PO4-Na2HPO4 pH 7.8, 2 × Denhardt’s and 0.5% SDS at room temperature. Filters were exposed for autoradiography as described above.

**R-Loop hybridization and data analysis.** The R-loop hybridization reaction mixture (Thomas et al., 1976) contained 1 × R-loop salts (0.1 M-HEPES pH 7.8, 0.4 M-NaCl, 0.01 M-EDTA), 70% formamide (98%, Mallincrodt), La-immunoprecipitable HCMV RNA and 100 μg/ml of HindIII-digested pTH-G (a recombinant containing the HindIII G fragment of HCMV cloned into pBR322). After incubation in sealed tubes for 10 to 16 h at 55 °C, the hybridization mixtures were treated with 5% formaldehyde for 5 min on ice to stabilize the RNA–DNA hybrids (Grossman et al., 1961), and aliquots were diluted into hyperphases of 0.1 M-Tris-HCl pH 8.5, 0.01 M-EDTA, 50 μg/ml cytochrome c (Serva) and 40% formamide (Davis et al., 1971). These hyperphases also contained single-stranded φX174 (5386 bases) or single-stranded M13 (7238 bases) and double-stranded pBR322 (4363 bp) as length standards. The hyperphase was then spread over a hypophase of 0.01 M- Tris-HCl pH 8.5, 0.001 M-EDTA and 10% formamide (Davis et al., 1971). After 1 min, the protein film was picked up on Parlodion-coated 200-mesh copper grids, stained with 10-4 M-uranyl acetate, rotary-shadowed with evaporated platinum, and micrographs were taken at 11700 x magnification using a Philips 400 transmission electron microscope. Average values of double-stranded and single-stranded length standards were determined for micrographs from one grid, and these values were used to convert the lengths of RNA–DNA hybrids and DNA–DNA hybrids to bp.

**RESULTS**

**Immunoprecipitation of 32P-labelled RNA from uninfected and HCMV-infected cells**

To confirm that the La, Ro, Sm and (U1)RNP antisera used were monospecific, RNP complexes were immunoprecipitated from cells and extracted RNAs were analysed by electrophoresis through polyacrylamide gels. Before immunoprecipitation, RNAs were labelled in vivo with [32P]orthophosphate in HCMV-infected or uninfected MRC-5 cells until 48 h p.i. Fig. 1 shows the species of in vivo labelled small RNAs obtained. Normal sera (Fig. 1) or Pansorbin alone (data not shown) precipitated no RNAs from uninfected or HCMV-infected cells. There were no differences in the patterns of small 32P-labelled RNA species immunoprecipitated by La, Ro, Sm and (U1)RNP antisera from uninfected or HCMV-infected
Fig. 1. Small RNAs immunoprecipitated by SLE antisera from uninfected and HCMV-infected cells. Uninfected (lanes U) or HCMV-infected (lanes I) MRC-5 cell RNP complexes were immunoprecipitated after in vivo labelling with [32P]orthophosphate from 1 to 48 h p.i. 32P-labelled RNA was purified from these complexes, denatured, and 6000 c.p.m. per lane was electrophoresed through 5% polyacrylamide–7 M-urea gels (Maniatis & Efstratiadis, 1980). Lanes 1, normal human serum; lanes 2, La antiserum; lanes 3, Ro antiserum; lanes 4, Sm antiserum; lanes 5, (U1)RNP antiserum.

Identification of RNAs was based on migration relative to co-electrophoresed 32P-labelled (using the Klenow fragment of DNA polymerase I) BamHI-, BglI- and HindIII-digested pBR322 (lane M).

Substantial amounts of unresolved RNA present in the upper portion of electrophoresis lanes suggested that larger RNA species were also immunoprecipitated. These larger RNA species were resolved by electrophoresis through 1% agarose gels. Two cellular RNAs of 5’0 and 2’0 kb were immunoprecipitated by (U1)RNP, Sm, La and Ro antisera from both uninfected and HCMV-infected cells (Fig. 2). These two cellular RNAs were identified as the 28S and 18S rRNAs by probing Northern blots of unlabelled immunoprecipitated RNAs with 32P-labelled recombinant plasmids (Gonzales et al., 1985; Gonzales & Schmickel, 1986) containing the
rRNA transcription units (data not shown). In addition, a novel 2.8 kb RNA was immunoprecipitated from HCMV-infected cells with La and Ro antisera. This 2.8 kb RNA was also recovered, although much less abundantly, by Sm antisera, but was never detected in (U1)RNP immunoprecipitates. A small amount of these cellular RNAs, but no infected cell-specific 2.8 kb RNAs, were immunoprecipitated by normal antisera (Fig. 2) or Pansorbin alone (not shown). These characteristic immunoprecipitation profiles were found when Protein A-Sepharose was substituted for Pansorbin, and when at least four different sources of La, Ro, Sm and (U1)RNP antisera were used.

Hybridization of HCMV DNA with immunoprecipitated RNA from infected cells

To determine whether the 2.8 kb infected cell-specific RNA might be viral, Southern hybridizations between *HindIII* or *XbaI* restriction enzyme fragments of HCMV DNA (prepared essentially as described in Kilpatrick & Huang, 1977) and immunoprecipitated *in vivo* 32P-labelled RNAs were performed (Fig. 3). RNAs immunoprecipitated by La, Ro and Sm antisera from HCMV-infected cells hybridized to *HindIII* restriction fragments E, G and K, and *XbaI* restriction fragments D, E, F and M (Fig. 3a). Comparison of the genomic positions of these fragments on *HindIII* and *XbaI* restriction enzyme maps (Fig. 3b) revealed that hybridization occurred specifically within the long terminal (TRL) and long internal (IRL) repeat sequences. RNA immunoprecipitated from uninfected cells, or from infected cells by (U1)RNP antisera, showed no hybridization to HCMV DNA (Fig. 3a).

The site of homology within the long repeat sequences was further defined with pTH-G, an HCMV recombinant containing essentially all the long repeat sequences. Southern hybridizations were performed between *BamHI*, *BglI* and *HindIII*-digested pTH-G and 32P-labelled RNAs immunoprecipitated by La, Ro and Sm antisera from HCMV-infected cells (Fig. 3b). The RNAs hybridized strongly to the *BamHI* 4.65 kb fragment of pTH-G and weakly to the adjacent *BamHI*-BglI 3.3 kb fragment. Thus, immunoprecipitated RNAs hybridized entirely within a 7.95 kb region of the HCMV long repeats (within coordinates 0.7973 to 0.8306 of the IRL and coordinates 0.0005 to 0.034 of the TRL).
Fig. 3. Genomic regions of HCMV DNA homologous to RNAs immunoprecipitated by SLE antisera. (a) Southern blots of HindIII- (lanes H) or XbaI- (lanes X) digested HCMV DNA (Towne strain) were probed with $^{32}$P-labelled RNAs (2 x $10^5$ c.p.m.) immunoprecipitated by Sm, (U1)RNP, La, or Ro antisera (lanes 1 and 5, 2 and 6, 3 and 7, 4 and 8, respectively) from uninfected (lanes 1 to 4) or HCMV-infected (lanes 5 to 8) cells at 48 h p.i. Lanes H and X without an indicated antiserum are $^{32}$P-labelled DNA fragments of the restriction enzyme-digested HCMV DNA. Letters on the left and right of infected sample lanes indicate hybridizing HindIII fragments and XbaI fragments, respectively. (b) Hybridization of immunoprecipitated RNAs to the Towne HindIII G fragment. HindIII and XbaI restriction maps are adapted from LaFemina & Hayward (1980) with arrows indicating the prototype orientation. The HindIII G fragment was cloned into pBR322 to form the recombinant designated pTH-G and the restriction enzyme cleavage sites for HindIII (i), BamHI (●) and BglI (●) were determined using multiple enzyme digests. The thickness of the bar indicates relative hybridization of the immunoprecipitated RNAs with each restriction fragment of pTH-G.
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Fig. 4. Northern analysis of immunoprecipitated HCMV RNAs homologous to the long repeats of HCMV. RNAs were immunoprecipitated from HCMV-infected cells with La, Ro or Sm antisera or from mock-infected cells with La antiserum at 48 h p.i., purified, electrophoresed through 1% agarose gels, blotted, and probed with $^{32}P$-labelled pTHG-4.65 DNA. T, total infected cell RNA; MI, mock-infected cell RNAs immunoprecipitated with La antiserum. Lanes 1, 2 and 3 contain RNAs immunoprecipitated at the first, second and third circles of antisera, respectively. Sizes of RNAs are indicated in kb.

**Northern analysis of immunoprecipitated RNAs**

To determine whether other HCMV RNAs in addition to the abundant 2.8 kb RNA were immunoprecipitated, Northern hybridizations were done. In these experiments, Northern blots of RNAs immunoprecipitated from infected and mock-infected cells were probed with either whole HCMV DNA, pTH-G or pTHG-4.65 (a recombinant containing the BamHI 4.65 kb fragment of strong homology shown in Fig. 3b cloned into pBR322). Analysis of RNAs immunoprecipitated throughout HCMV infection (0 to 72 h) demonstrated that HCMV RNAs were immunoprecipitated most abundantly at late (48 to 72 h) times p.i. In Fig. 4, viral RNAs immunoprecipitated with La, Ro and Sm antisera from HCMV-infected cells at 48 h p.i. are shown. The blots are purposely over-exposed to reveal the minor hybridizing species. It can be seen that in addition to the abundant 2.8 kb RNA in profiles of $^{32}P$-labelled RNAs, minor RNAs of 2.5, 1.9, 1.45 and 1.3 kb are detected. These five RNA species were each immunoprecipitated by La and Ro antisera, and less abundantly by Sm antisera. No hybridization was detected to RNAs immunoprecipitated from mock-infected cells. In total RNA profiles, a relatively abundant 5.6 kb, as well as the immunoprecipitable 2.8 kb RNA, were routinely detected. However, the 5.6 kb RNA was never found to be immunoprecipitated by any of the antisera.

In the experiment shown in Fig. 4, immunoprecipitation of the 2.8 kb RNA was titrated via three successive cycles of immunoprecipitation to determine whether immunoprecipitation by each of the antisera was quantitative. The majority of the 2.8 kb RNA was immunoprecipitated during the first cycle of immunoprecipitation indicating that immunoprecipitation is quantitative. Probing of these Northern blots with whole HCMV DNA, pTH-G and pTHG-4.65 gave identical results, indicating that these RNAs each hybridize to the long repeat sequences identified by Southern analysis.
The observation that 28S and 18S rRNAs are immunoprecipitated by (U1)RNP, Sm, La and Ro antisera (Fig. 2) suggested that the 2.8 kb infected cell-specific RNA might be polysome-associated. Northern blots of either La immunoprecipitated (Fig. 5, lane 1) or polysomal (Fig. 5, lane 2) RNA isolated from HCMV-infected cells at 48 h p.i. were probed with ^32^P-labelled whole HCMV DNA. It can be seen that the immunoprecipitated 2.8 kb RNA (and the minor 2.5, 1.45 and 1.3 kb RNAs) were also detected in the polysomal fraction. The minor 1.9 kb RNA immunoprecipitable by La antisera was not detected in the polysomal fraction, perhaps due to increased hybridization in that region. The 2.8 kb viral RNA is a major late viral RNA associated with polysomes. However, not all viral RNA species that are polysome-associated are immunoprecipitable. For example, the 6.8 and 4.8 kb polysomal viral RNAs were never detected in immunoprecipitates.

As an additional control to ensure that immunoprecipitation was specific to a distinct subset of HCMV RNAs, immediate early (IE) RNAs (from anisomycin-treated cells harvested at 12 h p.i.) were immunoprecipitated with either La, Ro or Sm antisera. When Northern blots were probed with whole HCMV ^32^P-labelled DNA, the total RNA profile consisted of two abundant RNAs of 5.0 and 1.95 kb as well as several other minor species. The 5.0 and 1.95 kb RNAs are the two most abundant IE polysome-associated RNAs and are encoded by sequences within the long unique region of the genome (Wathen & Stinski, 1982). However, as determined by densitometry, less than 5% of these IE RNAs was found in La and Ro immunoprecipitates and less than 10% was recovered in Sm immunoprecipitates (data not shown). The RNAs were not degraded during the immunoprecipitation procedure since the majority of both the 1.95 and 5.0 kb RNAs were recovered in the supernatants.

A possible concern was that immunoprecipitation of viral RNAs might be the result of a
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Fig. 6. Inhibition of viral RNA immunoprecipitation by La antigen. Increasing amounts of partially purified La antigen preparation or RNase-free BSA were added to infected cell sonicates, and RNAs were immunoprecipitated, blotted and probed with \(^{32}\)P-labelled pTHG-4-65; lane 1, no La antigen or BSA; lane 2, 0.25 mg La antigen and 7.75 mg BSA; lane 3, 0.5 mg La antigen and 7.5 mg BSA; lane 4, 1 mg La antigen and 7 mg BSA; lane 5, 2 mg La antigen and 6 mg BSA; lane 6, 4 mg La antigen and 4 mg BSA; lane 7, 8 mg La antigen, no BSA; lane 8, no La antigen, 8 mg BSA.

minor antigenic specificity or anti-RNA activity peculiar to certain lots of La, Ro or Sm sera. However, at least four different lots of La, Ro, Sm and (U1)RNP sera were used in immunoprecipitation experiments, and the results were always identical. In addition, the ability of other autoimmune antisera to immunoprecipitate these viral RNAs was tested. When anti-DNA, anti-centromere, anti-nucleolus or anti-(U1)RNP antisera were used, less than 5% of the viral RNAs immunoprecipitated by La antisera was recovered by these other antisera (data not shown). In addition, when \(^{32}\)P-labelled infected cell RNAs extracted from La, Ro or Sm immunoprecipitates were incubated with either La, Ro or Sm antisera, respectively, it was found that these RNAs were not re-immunoprecipitable (data not shown). Thus, although there may be a low level of non-specific trapping (<5%), immunoprecipitation of the 2.8 kb viral RNA by La or Ro antisera cannot be accounted for by trapping of RNA in immunoprecipitates nor by anti-RNA activity in the sera.

Inhibition of viral RNA immunoprecipitation by the addition of purified La antigen to cell extracts

If immunoprecipitation of these viral RNAs is due to a specific association of the RNAs with cellular La and Ro antigens or RNP s, then addition of increasing amounts of one of these SLE antigens, such as La, to cell sonicates before immunoprecipitation should inhibit immunoprecipitation of these HCMV RNAs. Although silver-stained polyacrylamide gels indicated that the partially purified La antigen preparation (Immunovision) used in these experiments did not contain only the La protein, as determined by immunoblotting, only the La protein was recognized by La antisera (not shown). It was determined (by immunoblotting) that a maximum of 1.6 mg of the La antigen preparation was required to saturate the antigen-binding sites in 16 µl of La antiserum. Thus, in competition experiments, increasing amounts (0 to 8.0 mg) of La antigen or RNase-free BSA (such that the total protein concentration for each sample was constant) were added to equal amounts (1.2 x 10^7 cells) of cell sonicates and RNAs were immunoprecipitated (with 16 µl La antiserum) as usual.

Immunoprecipitation of the viral RNAs was increasingly inhibited on addition of La antigen (Fig. 6). Thus, 0.25 mg of La antigen, when added to infected sonicated cell material, inhibited the immunoprecipitation of the 2.8 kb viral RNA by approximately 80%, as quantified by densitometry, whereas higher concentrations of La antigen (0.5 to 8.0 mg) inhibited immunoprecipitation completely. There was some inhibition (20% as quantified by densitometry) of immunoprecipitation by 8 mg of BSA alone (Fig. 6, lane 8); however, this
inhibition was significantly less than that seen on addition of 0.25 mg La antigen (Fig. 6, lane 2). That competition by La antigen occurred at those concentrations determined to be saturating for the amount of La antiserum added shows that immunoprecipitation of these viral RNAs by La antiserum reflects an association between the RNA and cellular La antigens or RNPs, and is not the result of a minor specificity in the antiserum.

R-Loop mapping of viral RNAs immunoprecipitated by La antiserum

To determine the location of sequences that code for these immunoprecipitated RNAs, R-loop mapping to the HindIII G fragment [0-7904 to 0-8449 map units (m.u.)] of HCMV DNA was performed. R-Loops were oriented with respect to the genomic map by a secondary stem structure (see Fig. 7) which was consistently found at the 0.8449 m.u. end (as determined by Bg/II restriction enzyme analysis) of the HindIII G fragment. Five classes of abundant R-loops of 2.43, 2.14, 1.89, 1.51 and 1.29 kb in size were found, which by best-fit analysis were located to the same region of the HindIII G fragment, approximately 4800 bp from coordinate 0-7904 and

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Fig. 7. High resolution mapping of La-immunoprecipitable HCMV RNAs. Map coordinates of each R-loop are given and arrow indicates secondary stem structure. (a) 2.432 kb R-loop; (b) 2.142 kb R-loop; (c) 1.896 kb R-loop; (d) 1.511 kb R-loop; (e) 1.295 kb R-loop.
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6600 bp from coordinate 0.8449 (Fig. 7 and 8). The genomic location of each of the immuno precipitable RNAs is summarized in Table 1. The 2.8 and 2.5 kb RNAs as identified by Northern analysis appear to correspond to the 2.43 and 2.14 kb RNAs, respectively. To determine whether either of these RNAs were polyadenylated, which would explain their smaller size by R-loop analysis, RNAs immuno precipitated by La were oligo(dT)-selected (Maniatis et al., 1982) and then analysed in comparison with unselected La-precipitated RNAs by Northern analysis. Both the 2.8 and the 2.5 kb RNAs were recovered in the polyadenylated fraction (data not shown). The 1.9, 1.45 and 1.3 kb RNAs hybridized to the same DNA region as the 2.8 and 2.5 kb RNAs, but the coding region is the same size as the RNA size determined by Northern analysis (Table 1, Fig. 8).

To determine the direction of transcription of the RNAs precipitated by La (Fig. 8), the BamHI 4.65 kb fragment of the Towne strain of HCMV was cloned into the Gemini vector (Promega) such that each strand of the viral DNA could be independently transcribed from either the SP6 or the T7 bacteriophage promoter (as described by Promega). When the synthesized 32P-labelled transcripts were hybridized to Northern blots of La-precipitated RNAs, only the probe synthesized with the SP6 polymerase (which transcribed from coordinates 0.7973 to 0.8168 in a 5' to 3' direction) hybridized to the 2.8, 2.5, 1.9, 1.45 and 1.3 kb RNAs (not shown). Therefore, these immuno precipitable RNAs are transcribed from the BglI–BamHI 3.3 kb fragment into the BamHI 4.65 kb fragment (see Fig. 8). The 5' ends of the 2.8, 2.5, 1.9, 1.45 and 1.3 kb RNAs are separated by more than 100 bp (greater than the limit of resolution, Fig. 8), indicating that these RNAs do not have common 5' ends. The 2.8 and 2.5 kb RNAs may share 3' ends because these ends are less than 100 bp apart and thus cannot be distinguished as separate ends (Fig. 8; Table 1). The 3' ends of the 1.9, 1.45 and 1.3 kb RNAs, located between 0.8108 and 0.8110 m.u., are also separated by less than 50 bp which suggests that these 3' ends are common.
Table 1. Summary of map locations of immunoprecipitated HCMV RNAs*

<table>
<thead>
<tr>
<th>R-Loop size</th>
<th>Map location</th>
</tr>
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<tbody>
<tr>
<td>2432 ± 93</td>
<td>0-8093-0-8189</td>
</tr>
<tr>
<td>2142 ± 52</td>
<td>0-8097-0-8182</td>
</tr>
<tr>
<td>1898 ± 71</td>
<td>0-8109-0-8184</td>
</tr>
<tr>
<td>1511 ± 56</td>
<td>0-8108-0-8167</td>
</tr>
<tr>
<td>1292 ± 52</td>
<td>0-8110-0-8161</td>
</tr>
</tbody>
</table>

* The limit of resolution for R-loop measurements was calculated by averaging the standard deviations of all DNA markers, and was determined to be 100 bp. The positions of R-loops were displayed as blocks on graph paper and aligned to produce a best-fit pattern.
† Numbers in parentheses indicate the number of R-loops of that size in a total of 105 R-loops measured.

DISCUSSION

This study has shown that certain HCMV RNAs can be immunoprecipitated by La, Ro and, to a lesser extent, Sm antisera. The immunoprecipitated RNAs include an abundant 2.8 kb RNA, as well as the much less abundant 2.5, 1.9, 1.45 and 1.3 kb RNAs. These RNAs were all found to hybridize within a 7-95 kb region of HCMV long repeat sequences. All of these RNAs (2.8, 2.5, 1.9, 1.45 and 1.3 kb) are derived from the same genomic sequence (within 0.8093 and 0.8189 m.u.) and are transcribed in the same direction such that the 5' ends of the RNAs are 3045 to 3756 bp from the junction of the long and short repeat sequences (Fig. 8). It appears that the 5' ends of the 2.8, 2.5, 1.9, 1.45 and 1.3 kb RNAs are all distinct, whereas certain of the 3' ends (e.g. of the 2.8 and 2.5 kb RNAs) may be common, indicating that either transcription of each RNA is initiated at five different promoters, or that a larger RNA precursor, such as the 2.8 kb RNA is processed (or degraded) to generate the smaller species. It was of interest to determine whether HCMV produces any small RNAs analogous to the La-immunoprecipitable EBER and VA RNAs (about 160 to 170 nucleotides). Although certain small (90 to 500 nucleotide) HCMV RNAs (which map to the long repeat region) are immunoprecipitated by La and Ro, but not by Sm or (U1)RNP, antisera (unpublished observations), these smaller species were of very low abundance and, unlike the larger RNAs, were not quantitatively immunoprecipitated. Thus, there is nothing to suggest that HCMV produces small immunoprecipitable RNAs analogous to EBER or VA RNAs.

Although the 2.8 kb RNA is a predominant RNA at late times, immunoprecipitation of this and the other long repeat region RNAs did not appear to be dependent on relative abundance. Among the RNAs homologous to the long repeat sequences and present along with the 2.8 kb RNA, an abundant 5-6 kb viral RNA was never immunoprecipitated.

The immunoprecipitable long repeat RNAs described herein are polysome-associated. Certain autoimmune sera interact with proteins of the large ribosomal subunit (Elkon et al., 1986), which may explain immunoprecipitation of the 18S and 28S rRNAs by these antisera. However, immunoprecipitation of the HCMV long repeat RNAs does not appear to be the result of non-specific trapping of ribosomal components in that all four antisera [La, Ro, Sm and (U1)RNP] recovered the 18S and 28S rRNAs, whereas the HCMV RNAs were immunoprecipitated by La and Ro, but not by Sm or (U1)RNP antigens. In addition, not all the RNAs associated with polysomes were immunoprecipitated. For instance, at least two abundant late viral polysomal RNAs of 6-8 and 4-8 kb were never immunoprecipitated (Fig. 5). Also, the abundant IE polysomal RNAs of 1.95 and 5.0 kb were not immunoprecipitated by either La or Ro antigens, and of the total RNA less than 10% was found in Sm immunoprecipitates.

Immunoprecipitation of the 2.8 kb RNA was shown to be specific in that it could not be explained by anti-RNA activity in the sera and was not seen when either normal or other autoimmune sera [i.e. anti-(U1)RNP, anti-centromere, anti-DNA, anti-nucleolus or anti-DNA topoisomerase I] were used. By immunoblotting, we found that the amount of antiserum required for maximum immunoprecipitation of 2.8 kb RNA was equivalent to that required for immunoprecipitation of cellular La antigen (data not shown). In addition, immunoprecipitation of the 2.8 kb RNA was inhibited by the addition of saturating amounts of La antigen to cell
extracts. These results are indicative, therefore, that immunoprecipitation of the 2-8 kb RNA reflects an association between the 2-8 kb RNA and cellular antigen or RNPs, and is not the result of a minor specificity in these antisera.

Whether the immunoprecipitable RNAs described in this study are identical to, or precursor products of, previously described HCMV transcripts remains to be determined. Major late transcripts of 2-8 and 2-7 kb for HCMV strains Towne and AD169, respectively, which are each encoded by the long repeat regions have been described (McDonough et al., 1985; Wathen & Stinski, 1982). When Northern blots of La-immunoprecipitated Towne RNAs were probed with a cDNA clone of the abundant AD169 long repeat 2.7 kb RNA (kindly provided by J. D. Oram, P. S. Greenaway and G. W. G. Wilkinson, Public Health Laboratory Service, Salisbury, U.K.), a 2.8 kb RNA was detected. The sequence of this 2.7 kb AD169 long repeat RNA has been reported (Greenaway & Wilkinson, 1987) and, although the sequence contains an open reading frame, no corresponding protein product has been detected in vivo or in vitro. We also have been unable to detect polypeptide products from attempts to translate the immunoprecipitable long repeat RNAs in vitro (unpublished observation). Similar to the immunoprecipitable 2-8 kb RNA we have described, this AD169 RNA is not spliced and is polyadenylated and accumulates throughout infection. Thus it seems likely that the 2-7 kb AD169 RNA described by the above investigators is the same as the immunoprecipitable 2.8 kb RNA described herein.

The nature of the interaction between these viral RNAs and La, Ro and Sm RNPs is as yet unknown. Although a major late polysomal transcript, the 2-8 kb viral RNA does not appear to be spliced or translated. Thus, immunoprecipitation is probably not the result of its association with the Sm RNPs involved in HCMV processing. Since the 28S and 18S rRNAs are also immunoprecipitated, it is possible that these immunoprecipitates contain a subset of polysomes which contain the 2-8 kb viral RNA and La, Ro and Sm RNPs. In that both viral and cellular RNP complexes have been implicated in the post-transcriptional processing and/or translation of mRNA, it may be that these viral RNAs which associate with cellular La, Ro and Sm proteins (or RNPs) may similarly be involved in the post-transcriptional regulation of HCMV and/or cellular gene expression.

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