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# In vitro selection of novel RNA ligands that bind human cytomegalovirus and block viral infection

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## ABSTRACT

**Ribonuclease-resistant RNA molecules that bind to infectious human cytomegalovirus (HCMV) were isolated in vitro from a pool of randomized sequences after 16 cycles of selection and amplification. The two ligands (L13 and L19) characterized exhibited high HCMV-binding affinity in vitro and effectively inhibited viral infection in tissue culture. Their antiviral activity was also specific as they only reacted with two different strains of HCMV but not with the related herpes simplex virus 1 and human cells. These two ligands appeared to function as antivirals by blocking viral entry. Ultraviolet (UV) crosslinking studies suggested that L13 and L19 bind to HCMV essential glycoproteins B and H, respectively. Thus, RNA ligands that bind to different surface antigens of HCMV can be simultaneously isolated by the selection procedure. Our study demonstrates the feasibility of using these RNA ligands as a research tool to identify viral proteins required for infectivity and as an antiviral agent to block viral infection.**

**Keywords: antiviral; cytomegalovirus; in vitro selection; RNA aptamer; RNA ligand**

## INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous herpes virus that causes mild or subclinical diseases in immunocompetent adults but may lead to severe morbidity or mortality in neonates and immunocompromised individuals (Britt & Alford, 1996; Mocarski, 1996). Infection by this virus accounts for one of the most common opportunistic diseases (i.e., CMV retinitis) encountered by AIDS patients. Very few effective drugs (e.g., ganciclovir) are currently available and the emergence of drug-resistant strains of HCMV has created a need for the development of new drugs and novel treatment strategies (Jacobson et al., 1991; Palestine et al., 1991).

In vitro selection or SELEX (systematic evolution of ligands by exponential enrichment) procedures (Ellington & Szostak, 1990; Tuerk & Gold, 1990; Joyce, 1992) have been used to isolate oligonucleotide molecules (aptamers) with high affinity to a wide variety of low-molecular-weight targets and large complexes such as red blood cell membranes (Ellington & Szostak, 1992; Gold et al., 1995, 1997; Pan et al., 1995; Morris et al., 1998; Yang et al., 1998; Homann & Goringer, 1999). This approach has also been used to select RNA aptamers to bind to Rous sarcoma virus (RSV) and African trypanosome, and RNA ligands that inhibited RSV infection were isolated (Pan et al., 1995; Homann & Goringer, 1999). In these procedures, single-stranded oligonucleotide molecules that exhibited the highest affinity to a target were selected from a pool of randomized sequences by reiterative cycles of selection and amplification. In this study, we employed a modified in vitro selection procedure to isolate ribonuclease-resistant RNA ligands that tightly bind to HCMV infectious particles. The selected ligands exhibited high HCMV-binding affinity in vitro and effectively inhibited viral infection in tissue culture. Two of the ligands appeared to block viral entry by interacting with viral surface glycoproteins. Our study demonstrates the feasibility of using these RNA ligands as a research tool to identify HCMV proteins essential for infectivity and as an antiviral agent to block HCMV infection.

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## RESULTS

### Isolation of ribonuclease-resistant RNA analogs that exhibit high affinity to HCMV

Our procedures (Fig. 1A; see Materials and Methods) to select RNA ligands involved the synthesis of RNA analog molecules that contained 2′ amino-modified py-

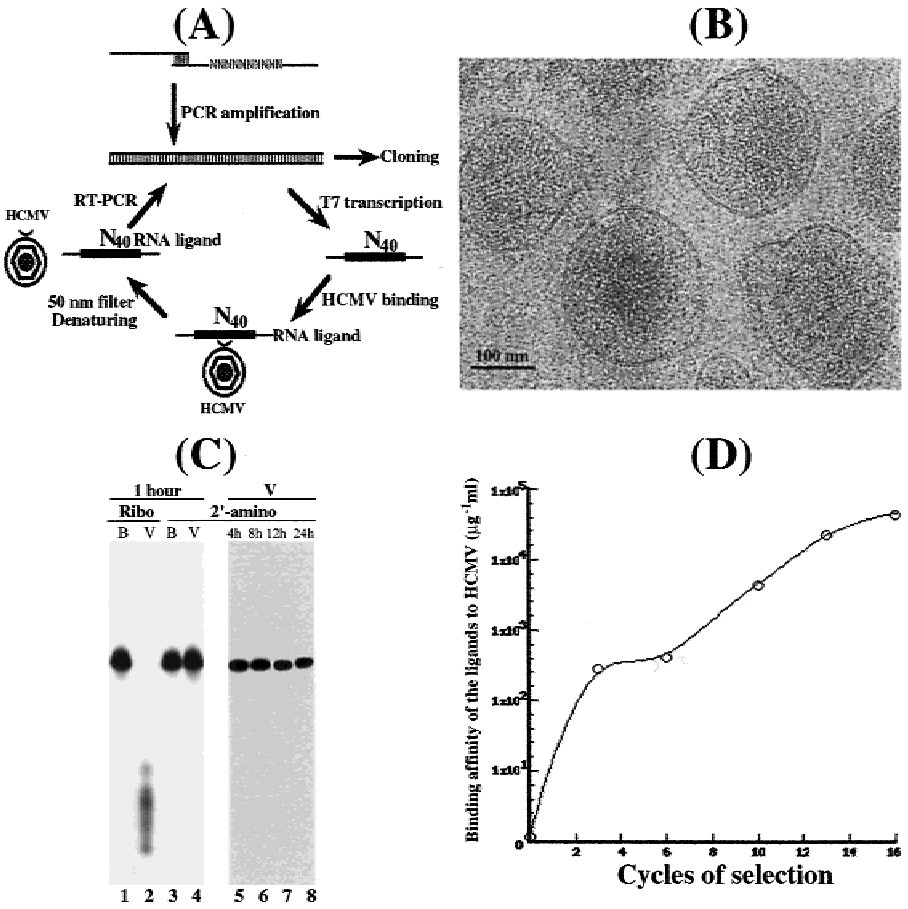


**FIGURE 1.** **A**: Schematic representation of the evolution in vitro procedures to select RNA analogs that bind to HCMV particles. The pool of DNA molecules contained a randomized sequence of 40 nt indicated as N. **B**: Electron cryomicrograph of the HCMV used in the selection experiments. **C**: Stability of the ligand molecules without modified nucleotides (Ribo, lanes 1–2) and with 2′-amino-modified pyrimidines (2′-amino, lanes 3–8) in the absence (lanes 1 and 3) and presence (lanes 2, 4–8) of HCMV. The 2′-amino-modified RNAs were synthesized as described in Materials and Methods. B: binding buffer III (20 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$) (lanes 1 and 3); V: DMEM culture media plus $1 \times 10^5$ pfu/mL HCMV and 10% fetal bovine serum (lanes 2, 4–8). 1 nM of radiolabeled $G_0$ ligand molecules was incubated under different conditions at 37 °C for either 1 h (lanes 1–4), 4 h (lane 5), 8 h (lane 6), 12 h (lane 7), and 24 h (lane 8), then subjected to separation in denaturing gels, and finally autoradiographed. **D**: Increased binding affinity of the populations of RNA analogs during selection from cycle 0 to cycle 16. Binding assays were carried out with different concentrations of virus and a trace amount (<100 fmol) of ligands. The values of binding affinity were calculated by dividing the percentage of bound ligands with the concentration of HCMV used ($\mu$g viral protein /mL). The protein assays of the infectious HCMV virions indicated that a stock of $1 \times 10^5$ pfu virus contained ~10 $\mu$g protein. Each point represents the mean of duplicate measurements.

rimidine nucleotides, selection experiments in vitro for the modified RNA molecules that bind to infectious HCMV, and purification and amplification of the selected sequences. The viral particles were isolated from HCMV-infected human foreskin fibroblasts (HFFs) by gradient ultracentrifugation as described previously (Irmiere & Gibson, 1985; Chen et al., 1999). The infectivity of the purified HCMV was evaluated by titering the particles in human fibroblasts and the intactness of their structures was examined first by obtaining negative staining images with a conventional transmission electron microscope and further confirmed by electron cryomicroscopy (Fig. 1B; Chen et al., 1999). Only those HCMV preparations that exhibited the highest titer [$>5 \times 10^8$ plaque forming units (pfu)/mL] and most intact particles were used for the selection. To increase the stability of the selected molecules, RNA analog molecules that contained 2′ amino-modified pyrimidines were synthesized in vitro by T7 RNA polymerase in the presence of the modified nucleotide triphosphates (Verma & Eckstein, 1998). RNA molecules with 2′ amino pyrimidines have been previously shown to be very stable in the presence of bovine and human serum and resistant to degradation by RNases (Pieken et al., 1991; Lin et al., 1994, 1996; Jellinek et al., 1995; Wiegand et al., 1996; Kubik et al., 1997; Lee & Sullenger, 1997; Pagratis et al., 1997). In our experiments, most of the 2′ amino-modified RNA molecules remained intact, even after a 24 h incubation with HCMV (Fig. 1C, lane 8), whereas the unmodified RNA molecules were totally degraded in 1 h (Fig. 1C, lane 2). Accordingly, the 2′ amino-substituted RNA molecules were used in the selection and were synthesized in vitro from a pool of DNA molecules that contained 40 randomized positions. Then these RNA analog molecules were allowed to bind to HCMV, and the mixtures passed through a 50-nm pore-size filter. Because HCMV is more than 200 nm in diameter (Fig. 1B; Chen et al., 1999), the bound ligands along with the viral particles were retained on the filter and subsequently purified whereas those unbound passed through. cDNA molecules were synthesized and amplified from the purified ligands and subsequently served as the templates for the synthesis of the ligand molecules to be used in the next round of selection (Fig. 1A). This process was repeated 16 times and the binding affinity (measured as the value of binding percentage/viral protein concentration) of the ligand population after 16 cycles was higher than that

of the initial randomized RNA ($G_0$) by a factor of more than $5 \times 10^4$ (Fig. 1D).

### Sequencing analyses and biochemical characterization of the selected ligands

Twenty eight sequences coding for the ligands after 16 rounds of selection were cloned and characterized (Table 1). These ligands were divided into six sets based on their primary nucleotide sequences. In sets II, III, and IV, the individual sequences were identical to each other within the same set. Meanwhile, each sequence in sets I and V either had the same sequence or extensive homology to other sequences of the same set. In contrast, the 12 sequences in set VI are unrelated to each other or to any other sets. The diversity of the selected sequences is consistent with the notion that these ligands might have been selected to recognize different epitopes or targets on the surface of HCMV. These selected sequences were not found in the genomic sequence of HCMV (Chee et al., 1990). This observation indicated that the selected sequences were neither derived from viral genomic sequence nor antisense to the HCMV sequences.

To analyze the relationship between the sequences of the selected ligands and their capabilities to bind HCMV, five ligands (L13, L17, L19, L49, and L57) were assayed for their binding affinities to the viral particles. These molecules were chosen as representatives of the most abundant selected sequences (Table 1). The protein assays of the infectious HCMV virions indicated that a stock of $1 \times 10^5$ pfu virus contained ~10 $\mu$g protein. A trace amount of radiolabeled ligand was incubated with different concentrations (pfu/mL) of purified HCMV in binding buffer III (20 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$) that was used in the selection. All five ligands exhibited high affinity to HCMV. More than 50% of the ligands were bound in the presence of $6 \times 10^5$ to $4 \times 10^6$ pfu/mL HCMV (Fig. 2A). In contrast, less than 10% of the $G_0$ molecules remained

**TABLE 1**. Partial sequences of twenty eight selected ligands. Only the sequence in the randomized positions are shown

| Clone # | The 40-nt-long randomized region |
|---|---|
| Set I | |
| 8 | TTACGGTCAC CTTACCCCTG GGTGTGCTCT TCCCGGTGGG |
| 13 | TTACGGTCAC CTTACCCCTG GGTGTGCTCT TCCCGGTGGG |
| 14 | TCACAGTCAC CTTACCCCTG GGTGTGCTCT TCCCGGTGGG |
| 55 | TCACAGTCAC CTTACCCCTG GGTGTGCTCT TCCCGGTGGG |
| 63 | TTACGGTCAC CTTACCCCTG GGTGTGCTCT TCCCGGTGGG |
| 70 | TCACAGTCAC CTTACCCCTG GGTGTGCTCT TCCCGGTGGG |
| Set II | |
| 1 | GCGAATTAAC ACATCGGGCC CATCGTCCGA GGTGCGTGGG |
| 57 | GCGAATTAAC ACATCGGGCC CATCGTCCGA GGTGCGTGGG |
| 69 | GCGAATTAAC ACATCGGGCC CATCGTCCGA GGTGCGTGGG |
| Set III | |
| 17 | CATCTCTCCT CACCATACCT CCACTTCCTG GGCTCGTGGG |
| 21 | CATCTCTCCT CACCATACCT CCACTTCCTG GGCTCGTGGG |
| Set IV | |
| 19 | CTCGAGCCAC CCCATAACCC TCAATACTCC AGGGATTGGG |
| 53 | CTCGAGCCAC CCCATAACCC TCAATACTCC AGGGATTGGG |
| Set V | |
| 11 | CATCACTTGA CCCTACTCTA CCTGGGCTGG ACTGGGTGGG |
| 58 | CTATTTCCCA CCCATATCCC CTTGGGCCCT TGGGTGTGGG |
| 49 | CTATATCCAC CCCATATCCC CTTGGCCCCT TGCGTGTGGG |
| Set VI | |
| 2 | GCACGACTCT CACTCAAGGG TCGATGCAGG CGTCTGTGGG |
| 3 | ACCTCCATGT CAATATCATC AGTATCAAAA TGGGTGCTGGG |
| 4 | AACCAACTTT TTTCAAACAC TCACTATCTG GGTGTATGGG |
| 5 | CACTCCTTCG CAACACCACT CACCTTGGGA CCTTGGGTGG |
| 9 | GCACCGACTC TCACTCAAGG GTCGATCAGG CGTCTGTGGG |
| 52 | ATGACTGACT ACACATGCCC CTTAGGGATG TATCTTAGGG |
| 56 | AACCAACCTC TCTCAAACCC TCACTATCGG GTTGTATGGG |
| 61 | ACCAGACGTA TCCACACTCA TTGGGCTTGG TCTCCGTGGG |
| 62 | CTACTCCCTC CCTAACCCTG GGTCCGCTAT ACATGGTGGG |
| 64 | GCCGAATTCA CACATCGGGC CCATCGTCGA GGTGCGTGGG |
| 65 | ACCCCTCTGC CTCACTCCAA TTCAGCGGGC GGTTCGTGGG |
| 18 | GGTCCTACGG ACTTTGGCAC GCAATCACTA GGTGTTTGGG |

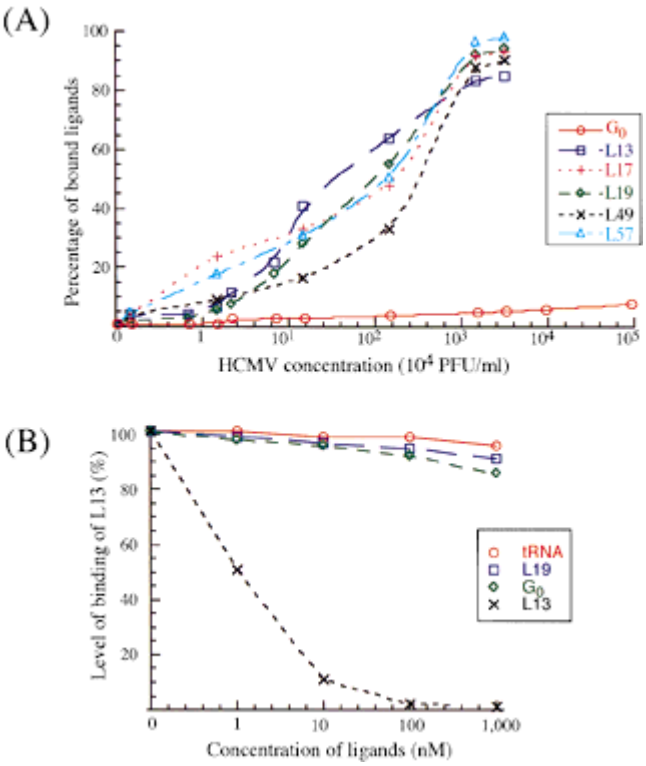


**FIGURE 2.** Binding affinity of the selected ligands to HCMV. **A**: 1 nM of different selected ligands were allowed to bind to different concentrations of HCMV particles. The values for the percentage of binding represent the mean of triplicate experiments and are not significantly different when 0.1–5 nM of ligands were used in the binding assays. **B**: 1 nM of radiolabeled L13 was allowed to bind to $1 \times 10^5$ pfu/mL (~10 $\mu$g viral protein/mL) HCMV in the presence of different concentrations of nonradiolabeled L13, L19, $G_0$, and $tRNA^{ser}$. The level of binding of L13 was calculated as the ratio of the percentage of bound radiolabeled L13 in the presence of other ligands over that obtained in the absence of these ligands. The values are the means of triplicate experiments. A value of 100% indicated that there was no competition between the binding of L13 and the other ligand molecules to HCMV.

bound even in the presence of $1 \times 10^9$ pfu/mL HCMV. The binding affinities of most of these ligands are at least $5 \times 10^4$ higher than that of the initial randomized pool (Figs. 1D, 2A). Ligands L13, L17, and L19 also exhibited similar binding affinities when binding assays were carried out in Dulbecco's modified Eagle media (DMEM), which resembled the physiological buffer conditions (data not shown). RNase A digestion of the ligands before binding resulted in the loss of the binding capability of these molecules, indicating that the intact structure of the ligands is essential for HCMV binding.

If L13 and L19 recognize different HCMV targets or epitopes, it is expected that these two ligands would not compete for binding to the viral particles. Binding affinity of L13 to HCMV in the presence of different concentrations of L19 (Fig. 2B) and vice versa was determined. As controls, the binding of L13 to HCMV was also assayed in the presence of $G_0$ RNA and $tRNA^{ser}$. The affinity of L13 to HCMV was not significantly affected in the presence of different concentrations of L19, suggesting that these two ligands recognize different viral epitopes or targets (Fig. 2B). Similar results were also observed when the binding of L19 to HCMV was assayed in the presence of L13 (data not shown). As expected, $G_0$ RNA and $tRNA^{ser}$ did not affect the binding of L13 to HCMV.

### Antiviral activity of the selected ligands

To determine whether the binding of selected ligands can interfere with the viral replication process and block infection, the purified HCMV particles were first preincubated in vitro with different concentrations of ligands 13, 17, 19, and $G_0$ to allow binding, and then used to infect HFFs at MOI of 0.001–0.01. After absorption for 2 h, cells were washed with culture medium (DMEM) to remove residual virus. Then the cells were overlaid with 0.5% agarose and incubated at 37 °C, and the number of viral plaques was counted at 10–14 days postinfection. Significant reduction of viral plaque formation was observed in cells infected with viruses that were pretreated with L13 and L19 but not with L17 and $G_0$ (Fig. 3A and data not shown). The observed level of inhibition of viral plaque formation by L13 and L19 was dependent on the concentrations of the ligands. Results from triplicate experiments indicated that the concentrations ($IC_{50}$) of L13 and L19 for 50% inhibition of plaque formation of $1 \times 10^5$ pfu/mL HCMV are $125 \pm 20$ nM and $35 \pm 10$ nM, respectively (Fig. 3A).

The production of HCMV was also studied to determine whether viral growth is inhibited by binding of the ligands. Cells were first infected with viruses pretreated with L13, L17, L19 and $G_0$, and then further incubated at 37 °C to allow HCMV to grow. Within 36 and 72 h postinfection, virus stocks were prepared from the infected cultures (cells and culture medium together) and the pfu count was determined by measurement of the viral titer on human fibroblasts. A significant reduction in viral yield was observed in experiments with L13 and L19 but not L17 and $G_0$ (Fig. 3B; data not shown). The inhibition of viral yield was also ligand dose-dependent, with an $IC_{50}$ for L13 and L19 equal to $100 \pm 20$ nM and $35 \pm 7$ nM, respectively. To further determine the inhibitory effects of the ligands on viral growth, cells were infected at a MOI of 1 with HCMV that were treated with 800 nM of $G_0$ and L19, or without the ligands. Virus stocks were prepared from the infected cells at 1-day intervals through 5 days postinfection and the pfu count was determined by measurement of the viral titer in human fibroblasts (Fig. 4). After 3 days postinfection, a reduction of at least 100-fold in viral yield was observed in cells that were infected with HCMV treated with L19, whereas no significant reduction was found in those treated with $G_0$ (Fig. 4). A reduction of viral plaque formation and viral growth was also observed when HCMV was first bound to L13 and L19 and then washed to remove most of the unbound ligands before being placed on cells (data not shown).

The binding and antiviral activity of L13 and L19 appeared to be specific for HCMV. In vitro binding affinities of L13 and L19 for HCMV strain AD169 were similar to those for another HCMV strain, Towne, but were at least $10^4$-fold higher than those for herpes simplex virus 1 (HSV-1) strain F (data not shown). These ligands also inhibited plaque formation and growth of HCMV (Towne) with a level of activity similar to the AD169 strain (data not shown). In contrast, no inhibition was observed when L13 and L19 were used with HSV-1 (Fig. 3C,D). These results indicate that these two ligands specifically interact with molecules that are found commonly in different HCMV strains but not in HSV-1.

### Blockage of viral entry by the selected ligands

L19 was chosen as a representative to further study the antiviral mechanism of the selected ligands. Several experiments were carried out to investigate which step(s) of the viral lytic cycle is blocked by L19. First, expression of the viral major immediate-early protein IE72, which is among the first proteins expressed after infection, was determined by Western analysis (Fig. 5A). Human fibroblasts were infected with viruses that were pretreated with culture media (DMEM) alone or in the presence of $G_0$, L19, or nAb, which is a mixture of anti-HCMV glycoprotein B (gB) neutralizing antibodies (Qadri et al., 1992; Navarro et al., 1993). The infected cells were harvested at 72 h postinfection and proteins from the infected cells were separated electrophoretically on SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, and finally stained with anti-IE72 monoclonal antibody mAb1203 (Fig. 5A; Navarro et al., 1993). The IE72 expression was barely detected in cells infected with HCMV pretreated with 680 nM of

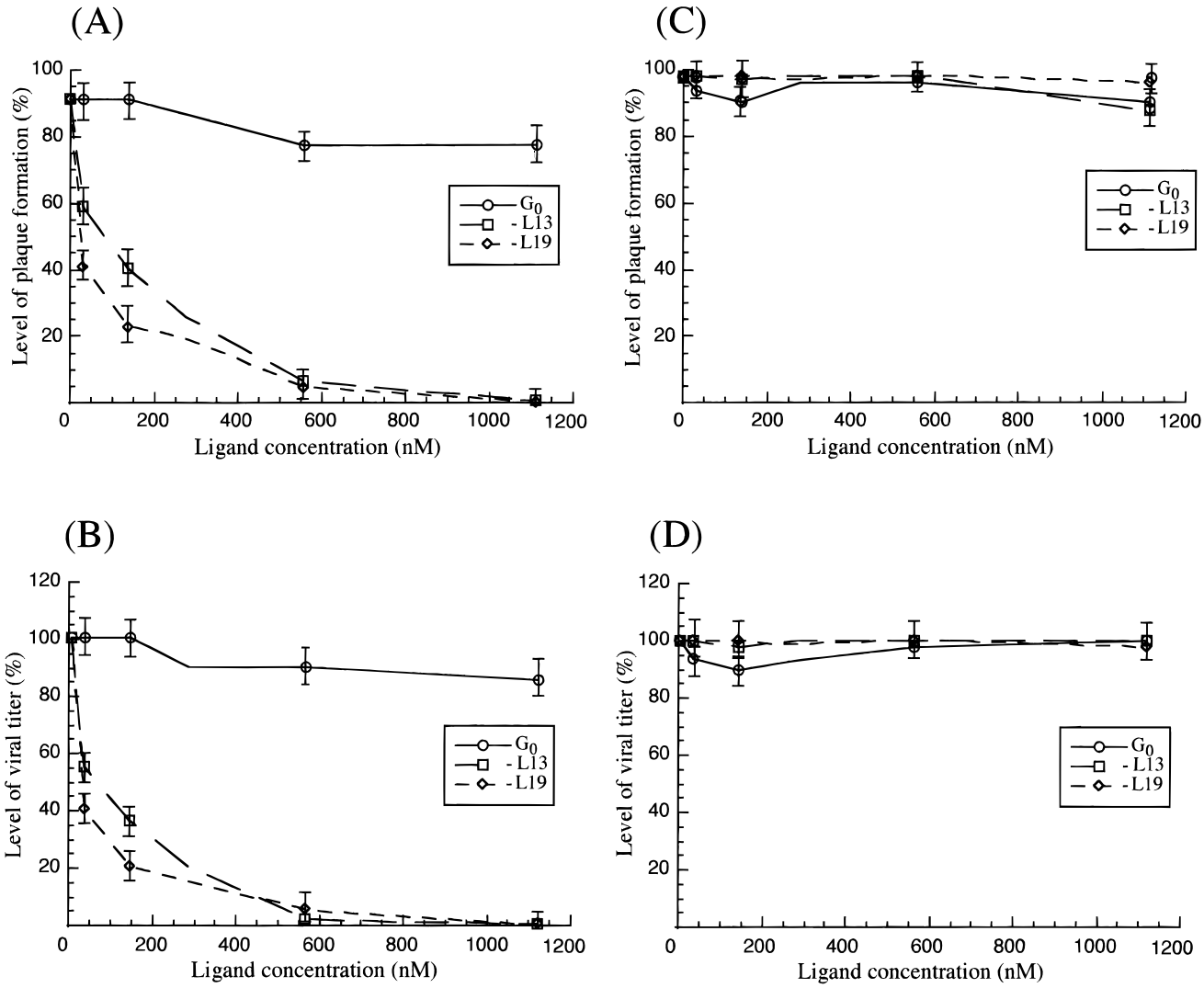


**FIGURE 3.** Effect of the ligands on plaque formation (**A** and **C**) and particle production (**B** and **D**) of HCMV (AD169) (**A** and **B**) and herpes simplex virus 1 (F) (**C** and **D**) in HFFs. $1 \times 10^5$ pfu/mL HCMV (AD169) or HSV-1 (F) were incubated in DMEM media alone or in the presence of different concentrations of $G_0$, L13, or L19 at 37 °C for 15 min before being used to infect HFFs at MOI of 0.005 (for plaque assay) or 0.3 (for titer assay). The levels of viral titer and plaque formation were calculated as the ratio of the titers and plaque numbers assayed from experiments with HCMV incubated in the presence of the ligands over those from experiments with HCMV incubated in DMEM alone, respectively. The values are the means of triplicate experiments.

L19 and 200 $\mu$g/mL of anti-HCMV gB neutralizing antibodies nAb (Fig. 5A, lanes 2 and 3; Navarro et al., 1993). In contrast, a substantial amount of IE72 protein was observed in cells infected with virus treated with either DMEM alone or in the presence of 680 nM $G_0$ RNA (Fig. 5A, lanes 1 and 4).

In the second set of experiments, cells were infected with HCMV pretreated with different concentrations of L19. Those cells that expressed IE72 were visualized by immunofluorescent microscopy with anti-IE72 antibody, mAb1203, and their numbers were counted (Fig. 5B). A significant reduction in the numbers of IE72-expressing cells was observed among cells infected with viruses pretreated with 680 nM L19 or 200 $\mu$g/mL anti-HCMV neutralizing antibodies nAb (Fig. 5Bc,e), whereas no reduction was observed in the presence of 680 nM $G_0$ (Fig. 5Bf). The level of reduction in the numbers of IE72-expressing cells was dependent on the concentrations of L19, with an $IC_{50}$ value of $35 \pm 7$ nM (Fig. 5C). These results suggest that L19 blocks a step prior to or during viral immediate-early gene expression, such as viral entry, uncoating, or transcription of IE72 mRNA. To distinguish among these possibilities, viral particles pretreated with different concentrations of L19 were incubated with human fibroblasts at 37 °C for 90 min to allow viral entry. Then the cells were washed extensively and further trypsinized to remove the unbound viral particles and those extracellular viruses that still attached to the cells. Total DNA was isolated from these infected cells and the level of intracellular viral genomic DNA was

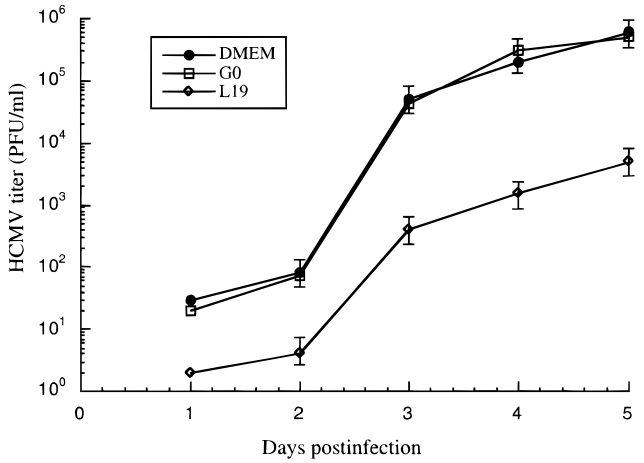


**FIGURE 4.** Growth curve analysis of HCMV in HFFs. $1 \times 10^5$ pfu/mL HCMV (AD169) were incubated in DMEM media alone (DMEM) or in the presence of 800 nM of $G_0$ ($G_0$) or L19 (L19) at 37 °C for 15 min before being used to infect HFFs at MOI of 1. Virus stocks were prepared from the infected cells at 1-day intervals through 5 days postinfection and the pfu count was determined by measurement of the viral titer on human fibroblasts. These values are the means from triplicate experiments. The standard deviation is indicated by the error bars. Error bars that are not evident indicate that the standard deviation was less or equal to the height of the symbols.

determined by PCR detection of the sequence of viral immediate-early gene IE1/IE2 (Fig. 6A). The level of human $\beta$-actin DNA was used as the internal control for the quantitation of HCMV DNA. A significant reduction in the level of intracellular HCMV DNA was observed in cells infected with virus treated with L19, indicating that the entry of the viral genome was blocked (Fig. 6, lanes 5 and 6). As a control, no HCMV DNA was detected in cells infected with viruses either pretreated with 200 $\mu$g/mL nAb or 50 $\mu$g/mL heparin, which blocked HCMV attachment (Fig. 6A, lanes 7 and 8; Compton et al., 1993; Mocarski, 1996). When viral infection was carried out at 4 °C to allow viral attachment but prevent penetration, intracellular HCMV DNA was barely detected (Fig. 6A, lane 9). These experiments strongly suggest that both the unbound viruses and those attaching to the cells were almost completely removed by the washing procedures and the HCMV DNA detected by PCR probably represented the viral genome that had entered the cells. Pretreatment of HCMV with 35 nM of L19 yielded a reduction of 55% in the level of viral DNA (Fig. 6B).

If inhibition of viral entry is the only block by L19 to a single cycle of viral replication, reduction of the input viral DNA and of the viral yield and gene expression should be quantitatively similar. Our results (Figs. 3–6) indicate that there is an excellent correlation among the level of reduction in the viral input DNA, the level of inhibition of IE72 gene expression, and viral production. For example, the treatment of HCMV with 35–40 nM of L19 yielded a reduction of 50–55% in the level of viral input DNA, the numbers of IE72-expressing cells, and the level of viral plaque formation and growth. Thus, L19 appeared primarily to block the viral entry step.

Similar experiments have also been carried out to determine which steps(s) of the viral lytic cycle is blocked by L13 (Figs. 5C and 6B). For example, the treatment of HCMV with 100 nM of L13 yielded a reduction of about 50% in the level of viral input DNA (Fig. 6B), the numbers of IE72-expressing cells (Fig. 5C), and the level of viral plaque formation and growth (Fig. 3A,3B). These results suggest that L13 also functions to block viral entry.

It is possible that L19 and L13 may block viral entry by interacting with a cellular surface receptor rather than a viral surface molecule. To distinguish between these possibilities, cells were preincubated with different concentrations of L19 or L13 (up to 3,000 nM) and then washed before being infected with HCMV. No reduction in the level of viral plaque formation or IE72 protein expression was observed (data not shown). Moreover, these ligands did not exhibit significant cytotoxicity. Cells that were treated with 3,000 nM L19 or L13 every other day for 30 days were indistinguishable from the untreated ones with respect to viability and plating efficiency at days 1, 2, 4, and 8 after seeding (data not shown). These observations suggest that the observed antiviral effects by L19 and L13 are due to their high affinity binding to the viral particles but not interactions with the cells.

### Identification of viral targets bound by L13 and L19

The protein assays of the infectious HCMV virions indicated that a stock of $1 \times 10^5$ pfu virus contained $\sim$10 $\mu$g of protein. When the complexes formed between HCMV and L13 or L19 were treated with proteinase K to remove most of the viral surface glycoproteins, the majority of the bound ligands were found to be dissociated from the viral particles (data not shown). This observation suggested that these ligands bind to viral proteins. To identify the viral targets to which L19 and L13 bind, the complexes formed between HCMV particles and radiolabeled L19 and L13 molecules were UV irradiated to allow crosslinking and then, separated on SDS-denaturing polyacrylamide gels. Those proteins that bound to L19 and L13 were expected to crosslink with the ligand and become radiolabeled. An example of the results is shown in Figure 7. Two crosslinked products with apparent molecule masses of about 170 and 55 kDa were found in the experiments with L19 and were immunoprecipitated by a monoclonal antibody specifically against HCMV glycoprotein B (gB) (Fig. 7, lanes 6 and 7). gB is one of the most abundant viral glycoproteins and is required for HCMV entry (Pereira et al., 1982; Britt & Auger, 1986; Cranage et al., 1986; Gretch et al.,

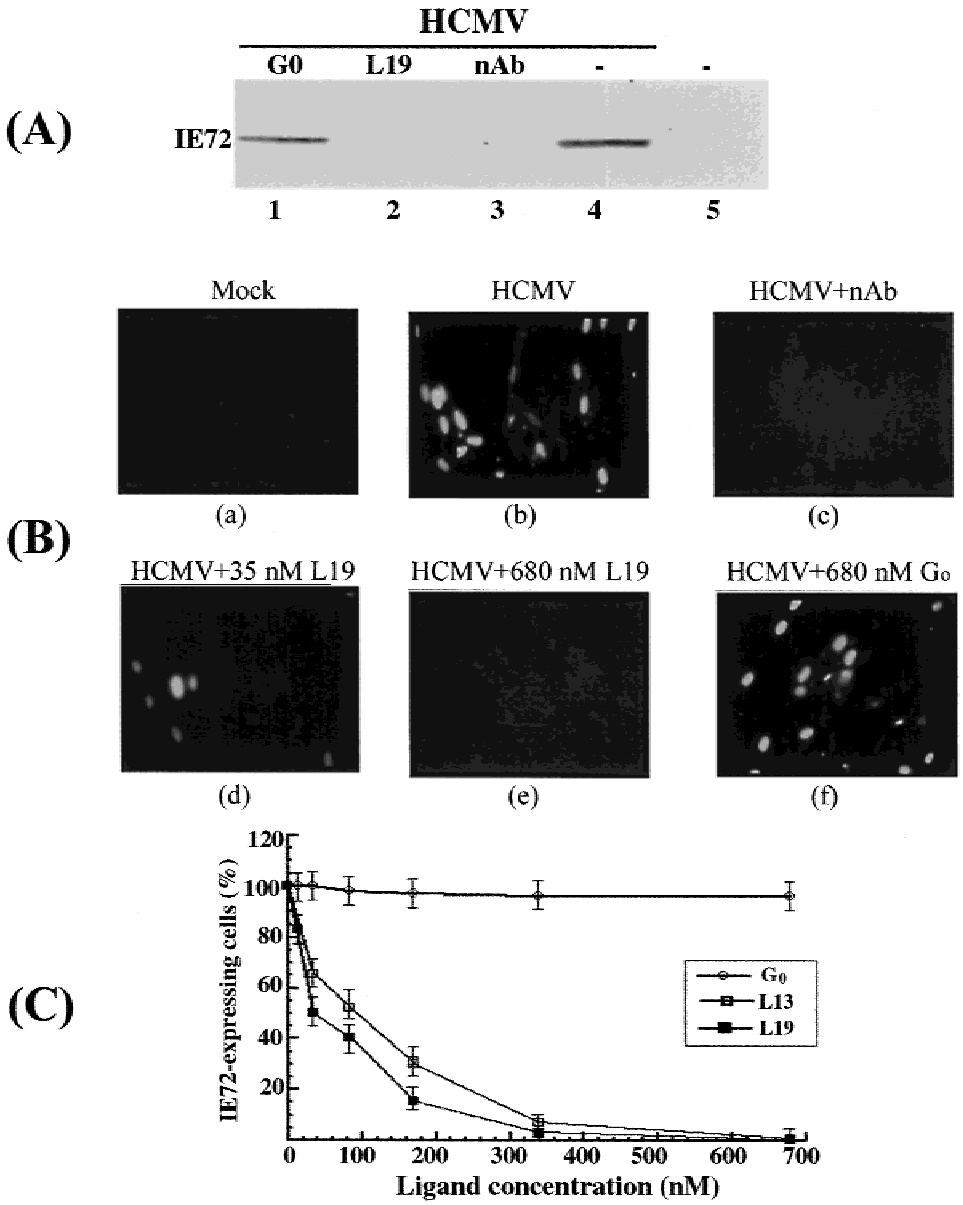


**FIGURE 5.** Expression of viral IE72 protein detected by Western analysis (**A**), immunofluorescent microscopy (**B**), and schematic representation of the numbers of IE72-expressing cells in the presence of ligands $G_0$ and L19 (**C**). **A**: The protein lysates were isolated from mock-infected cells (lane 5) or cells infected with viruses that were pretreated with DMEM alone (lane 4), $G_0$ (lane 1), L19 (lane 2), or a mixture of anti-HCMV gB neutralizing antibodies (nAb) (lane 3). The lysates were separated on SDS polyacrylamide gels, transferred to a membrane, and stained with anti IE72 monoclonal antibody mAb1203. The same amount of proteins (50 $\mu$g) was loaded on each lane. **B**: Cells were either mock-infected (a) or infected with viruses pretreated with DMEM alone (b) or in the presence of $G_0$ (f), L19 (d and e) or 200 $\mu$g/mL anti-HCMV gB neutralizing antibodies nAb (c). At 36 h postinfection, these cells were fixed and immunostained with anti-IE72 antibody mAb1203. **C**: The numbers of cells that expressed IE72 protein, as visualized in **B**, were counted. The values of the percentage shown were calculated by obtaining the ratio of the numbers of IE72-expressing cells infected with viruses treated with the ligands over those without the ligands. These values are the average derived from triplicate experiments. The antibodies used in **A** and **B** were in great excess of the detected antigens.

1988). The full-length gB protein has an apparent molecular mass of about 170 kDa and is proteolytically processed into an amino terminal product of 115 kDa and a carboxyl terminal product of 55 kDa (gp55) (Cranage et al., 1986; Spaete et al., 1988). Indeed, the crosslinked products comigrated with the gB products found in the HCMV particles (Fig. 7, lane 8). Therefore, L19 appeared to bind to gB. Meanwhile, a protein product with an apparent molecule mass of about 90 kDa was found to crosslink with L13 and was immunoprecipitated by a monoclonal antibody specifically against HCMV glycoprotein H (gH) (Fig. 7, lanes 14 and 15). gH is also a viral-abundant glycoprotein essential for HCMV entry and has an apparent molecular weight of 86 kDa (gp86) (Ras-

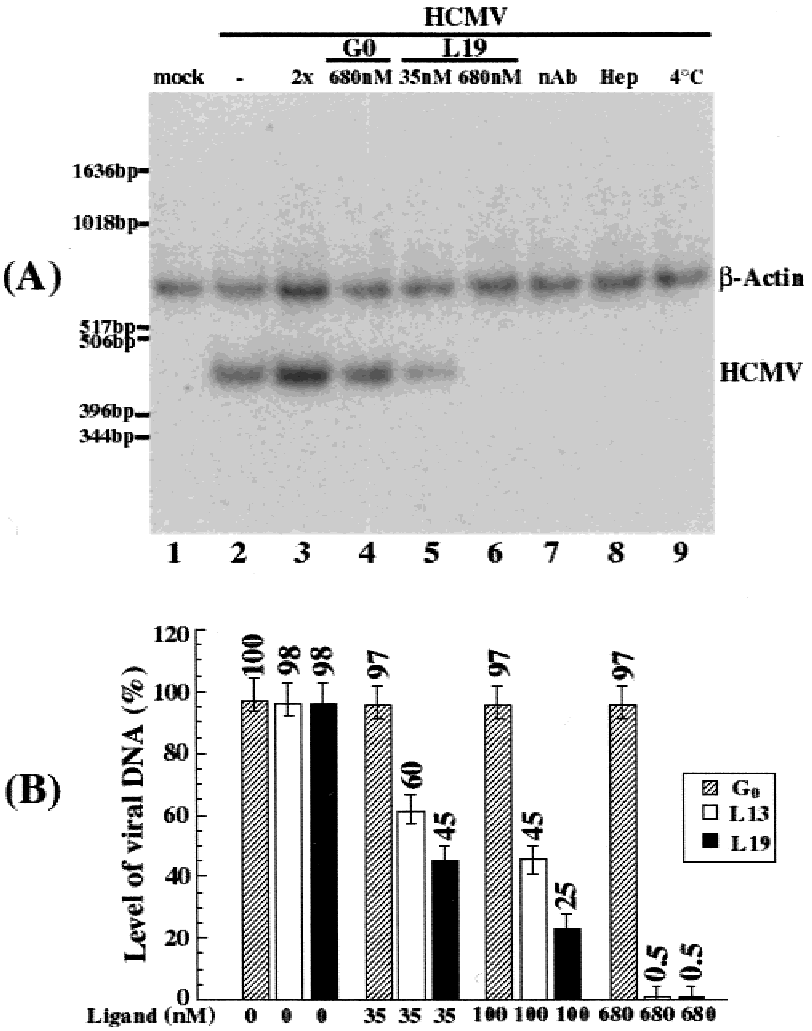


**FIGURE 6.** Level of intracellular HCMV genomic DNA during early viral infection. **A**: PCR was used to determine the level of viral DNA in cells that were mock-infected (lane 1) or infected with viruses pretreated with DMEM (lanes 2 and 3), $G_0$ (lane 4), L19 (lanes 5 and 6), a mixture of anti-HCMV gB neutralizing antibodies nAb (lane 7), and 50 $\mu$g/mL heparin (lane 8). In lane 9, viral infection was carried out at 4 °C to allow viral attachment but prevent penetration. Human $\beta$-actin DNA sequence was used as the internal controls. Twice the amount of the DNA templates was used in the PCR shown in lane 3 as in lane 2. The amplification by PCR was within the linear range. The radiolabeled PCR products were separated in 4% nondenaturing polyacrylamide gels and quantitated with a STORM840 PhosphorImager. **B**: Schematic representation of the level of viral DNA as assayed in **A**. The values were derived from triplicate experiments.

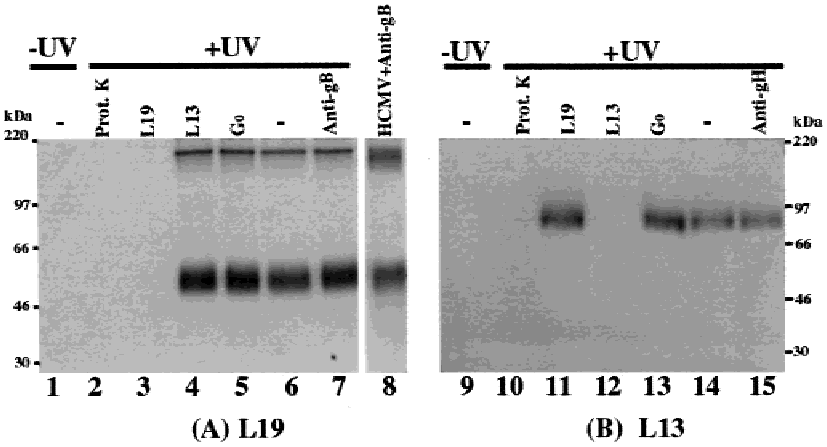


**FIGURE 7.** Autoradiograph of proteins that were crosslinked with L19 (**A**) and L13 (**B**) and separated on SDS-polyacrylamide gels. Radiolabeled L19 and L13 were mixed with HCMV in the absence (lanes 1–2, 6–7, 9–10, and 14–15) or presence (lanes 4 and 12) of a 20-fold molar excess of nonradiolabeled L13, L19 (lanes 3 and 11), or $G_0$ (lanes 5 and 13). The mixture was incubated in the absence (lanes 1 and 9) or presence (lanes 2–7, 10–15) of UV irradiation. In lanes 2 and 10, the crosslinked complexes were digested with proteinase K (200 $\mu$g/mL) before mixing with the disruption buffer (0.05 M Tris, pH 7.0, 8.5% [v/v] sucrose, 5% [v/v] $\beta$-mercaptoethanol, 2% [v/v] sodium dodecyl sulphate). In lanes 7 and 15, the protein samples were immunoprecipitated with monoclonal antibodies (anti-gB and anti-gH) specifically against HCMV-gB (lane 7) and gH (lane 15) before addition of the disruption buffer. In lane 8, Western analysis was carried out to stain the gB products of HCMV particles by an anti-gB monoclonal antibody. The molecular masses of the proteins in the Rainbow protein size marker (Amersham) are shown and given in kiloDaltons.

mussen et al., 1984; Gretch et al., 1988). The crosslinked conjugates with L19 and L13 were not observed either in the absence of the UV irradiation (Fig. 7, lanes 1 and 9) or when the crosslinked mixture was digested with protease K before being separated on SDS-polyacrylamide gels (Fig. 7, lanes 2 and 10). Moreover, the products crosslinked with L19 were not immunoprecipitated with the anti-gH antibody, whereas that crosslinked with L13 was not recognized by the anti-gB antibody (data not shown). The specificity of the crosslinks was also investigated in competition experiments with homologous and heterologous competitors. For example, the formation of the crosslinked species with L19 was blocked in the presence of the nonradiolabeled L19 but not L13 or $G_0$ (Fig. 7, lanes 3–5). These results suggest a specific interaction between L19 and these two gB products. Similar results were also observed to suggest that L13 specifically interacts with gH (Fig. 7, lanes 11–13).

## DISCUSSION

Nucleic acid-based ligands isolated from randomized sequences have been shown in vitro to exhibit high affinity and specificity to a wide variety of low-molecular-weight targets and large complexes such as red blood cell membranes (Ellington & Szostak, 1992; Gold et al., 1995, 1997; Pan et al., 1995; Morris et al., 1998; Yang et al., 1998; Homann & Goringer, 1999). More recently, this approach has also been used to select RNA aptamers to bind to intact infectious agents such as Rous sarcoma virus (RSV) and African trypanosome (Pan et al., 1995; Homann & Goringer, 1999). Moreover, the RNA ligands that bind to RSV have been shown to inhibit viral infection (Pan et al., 1995). Compared to monoclonal and polyclonal antibodies, nucleic acid-based ligands possess similar activity (high affinity and specificity) as well as other unique features (Gold et al., 1995). For example, the nature of the interactions (e.g., nucleic acid–protein interactions) between these ligands and their protein targets might be different from those (e.g., protein–protein interactions) used between antibodies and the same targets. Therefore, targets that are not considered immunogenic to antibodies may be tightly bound by RNA ligands. Using HCMV as a model system, we showed that ribonuclease-resistant RNA ligands were isolated from randomized sequences to bind HCMV particles and were very effective in blocking viral entry by interacting with different viral glycoproteins.

### Binding affinity and specificity of the antiviral RNA ligands

Several criteria have to be satisfied to use these RNAs as antiviral neutralizing agents. For example, their binding affinity and substrate specificity should be high, and their activity should be stable. In our study, the selected ligands exhibited a high affinity to HCMV particles and were highly effective in inhibiting viral production. Most of our ligands bound to the target tightly, and it only required 35–50 nM of L19 to interact with $1 \times 10^5$ pfu/mL viral particles and inhibit infection by 50% (Figs. 3–6). The binding affinity of the ligands also appeared to correlate with their activity in inhibiting viral infection. For example, the $G_{16}$ ligands exhibited higher affinity to HCMV and were more effective in blocking viral infection than the $G_{10}$ and $G_0$ ligands (data not shown). Moreover, ligands with higher binding affinity can be further isolated by increasing the stringency for binding during the selection. Meanwhile, consistent with previous observations (Pieken et al., 1991; Lin et al., 1994, 1996; Jellinek et al., 1995; Wiegand et al., 1996; Kubik et al., 1997; Lee & Sullenger, 1997; Pagratis et al., 1997), the 2′ amino pyrimidine modification significantly increases the stability of the selected ligands. These 2′-amino-substituted RNA ligands were very stable in culture media that contained HCMV and bovine serum (Fig. 1C).

Our results from three different sets of experiments also suggest that the selected ligands are highly specific. First, L13 and L19 did not compete for binding to HCMV (Fig. 2B). These results are consistent with our suggestion that L13 and L19 specifically recognize different viral proteins (i.e., gH and gB) required for infection. Second, L13 and L19 blocked viral growth in cells that were infected with two different strains of HCMV, but not with the related HSV-1. These ligands in vitro exhibited $10^4$-fold less binding affinity to HSV than to HCMV (data not shown). HSV and HCMV share several homologous glycoproteins, three of which (gB, gH, and gL) have been shown to be required for viral infectivity and entry (Mocarski, 1996; Roizman & Sears, 1996). Our observations suggest that these ligands interact with unique viral epitopes found in the gB and gH of HCMV but not of HSV. Finally, the selected ligands exhibited little cytotoxicity. When HFFs were treated with 3,000 nM of L19, a concentration four times higher than that required to abolish the infection of $1 \times 10^5$ pfu/mL of HCMV, they are indistinguishable from the untreated ones with respect to viability and plating efficiency. Moreover, their protein expression profiles were the same and no morphological differences were detected (data not shown).

### Mechanism of the antiviral activity of the selected ligands

None of the sequences of the selected ligands has extensive homology to both strands of the HCMV DNA genomic sequence, suggesting that the selected ligands do not function as antisense agents. Inhibition of viral infection was observed when HCMV was either preincubated with the ligands before being introduced

to the cells or was cointroduced to the cells with the ligands (Fig. 3; data not shown). Several lines of evidence suggest that L19 and L13 inhibit viral production and plaque formation by primarily blocking the entry of viral genome. The level of intracellular viral DNA, determined by PCR, probably reflects that of viral input genome during entry. Our data indicated that there is an excellent correlation among the level of reduction in the viral input DNA, the level of inhibition of IE72 gene expression, and viral production. These results strongly suggest that the inhibition of the entry of the viral genome into the cells is primarily responsible for all the observed antiviral effects by L19 and L13.

L19 and L13 crosslink predominately with two proteins of about 170 kDa and 55 kDa, and a single protein of approximate 90 kDa, respectively. These proteins were immunoprecipitated by anti-gB and anti-gH antibodies, suggesting that they are gB and gH. This notion is further supported by the fact that the size of these proteins are consistent with those of gB (170 and 55 kDa) and gH (86 kDa). Our observations that only these proteins but not other proteins were found predominately in the crosslink complexes also suggest that L19 and L13 primarily interact with gB and gH, respectively.

gB and gH are among the most abundant glycoproteins on the surface of HCMV. These two proteins are essential for viral entry and neutralizing antibodies that block viral infection have been isolated (Rasmussen et al., 1984; Cranage et al., 1986; Gretch et al., 1988; Pachl et al., 1989; Mocarski, 1996; Li et al., 1997; Huber & Compton, 1998). It is possible that L19 and L13 bind to novel neutralizing epitopes of gB and gH that are different from those identified by antibodies, given the fact that these ligands recognize their targets through RNA–protein interactions. This notion is further supported by the fact that binding of L19 to HCMV is not affected by the presence of three different anti-gB neutralizing antibodies (data not shown). Meanwhile, we cannot exclude the possibility that these ligands have additional contacts with the surface of HCMV that may also be important for viral infectivity.

### Generation of ligands as a research tool and as a therapeutic agent

The in vitro selection procedure has provided a powerful approach to isolate RNA ligands that bind to intact infectious agents (Pan et al., 1995; Homann & Goringer, 1999). Isolation of RNA ligands that target several different strains of a single agent or multiple agents can be easily accomplished by combining these agents together in the selection. Using a filter with a maximum pore size of 50 nm in the selection procedure will further facilitate the selection of RNA ligands with high affinity to almost any infectious agents. Crosslinking procedures with the selected ligands, as described in this and other studies (Morris et al., 1998; Homann & Goringer, 1999), should facilitate rapid identification of the targets of the infectious agents required for infectivity. Our result that the selected ligands bind to gB and gH further demonstrates the utility of the selection procedure to isolate ligands that recognize different abundant protein targets on the surface of an infectious agent.

HCMV, one of the largest human viruses, has the coding capacity for more than 220 open reading frames, 57 of which have been predicted to encode membrane proteins (Chee et al., 1990; Mocarski, 1996). To date, however, fewer than 10 virion envelope glycoproteins have been mapped to the viral genome and only four of them (gB, gH, gL, gO) have been implicated to be essential for viral entry (Rasmussen et al., 1984; Cranage et al., 1986; Gretch et al., 1988; Pachl et al., 1989; Mocarski, 1996; Li et al., 1997; Huber & Compton, 1998). Further studies using the selected ligands may lead to identification of new viral surface proteins that are important for infectivity. We note, however, that some of the selected ligands did not exhibit significant antiviral activity in spite of their high affinity to HCMV (e.g., L17). One of the possible explanations is that the viral targets or epitopes to which they bind are not essential for HCMV infectivity or replication in fibroblasts. It is possible that the binding of these ligands may interfere with HCMV infection in human cell types (e.g., monocytes) other than fibroblasts. Further studies using different cell types will reveal whether this is the case and may facilitate the identification of HCMV glycoproteins that mediate viral entry into different cell types. Identification of new viral essential glycoproteins will further our understanding of HCMV infection and provide novel targets for drug development. Moreover, these studies will greatly facilitate the development of these ligands as tools to study the biology of viral infection and as therapeutic agents for antiviral applications.

## MATERIALS AND METHODS

### Viruses, cells, and antibodies

HCMV (AD169 and Towne strain) and HFFs were obtained from American Tissue Culture Collection (ATCC; Rockville, Maryland) and Clonetics Inc. (San Diego, California), respectively. The HFFs were maintained and propagated in DMEM supplemented with 10% fetal bovine serum. HSV-1 (F), a prototype of human herpes simplex virus 1, was a gift from Dr. Bernard Roizman of the University of Chicago. The anti-IE72 monoclonal antibody, mAb1203, and a mixture of anti-HCMV glycoprotein B (gB) neutralizing monoclonal antibodies (nAb), mAb1204, 1206, and 1212, were obtained from Goodwin Cancer Research Institute (Plantation, Florida; Navarro et al., 1993). The monoclonal antibody against gH was purchased from Biodesign Inc. (Kennebunk, Maine).

HFFs were infected with HCMV and the viral particles were purified by double tartrate glycerol ultracentrifugation procedures (Irmiere & Gibson, 1985; Chen et al., 1999). The infectivity of the purified HCMV was evaluated by titering the particles in HFFs and the intactness of their structures was examined first by obtaining negative staining images with a conventional transmission electron microscope (University of California–Berkeley Electron Microscopy Core Facility) and further confirmed by electron cryomicroscopy (Chen et al., 1999).

## In vitro selection procedure

Double-stranded DNA templates were synthesized by PCR using oligonucleotide JH101 (5′-GCCGGATCCGGGCCTCATGTCGAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTGACCGTTTATTCTTGTCTCC-3′) as the template. JH101 contained a randomized sequence indicated as $(N)_{40}$ and underlined. The 5′ and 3′ PCR primers were oligodeoxynucleotides JH1031 (5′-CCGAAGCTTAATACGACTCACTATAGGGAGACAAGAATAAACGGTCAA-3′) and JH1052 (5′-CCCTCATGTCGAA-3′), respectively. JH1031 also contained the promoter sequence for bacteriophage T7 RNA polymerase. Transcription reactions were carried out in 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 0.5 mM each of ATP and GTP, and 1 mM each of 2′ amino-UTP and 2′ amino-CTP (Amersham Inc., Arlington Heights, Illinois) in the presence of $\alpha$-[$^{32}$P]-GTP and T7 RNA polymerase for 16 h at 37 °C. The synthesized RNA ligands were further purified in denaturing 8% polyacrylamide gels and then denatured in buffer I (20 mM Tris, pH 7.5, 100 mM NaCl) at 90 °C for 3 min. The RNA ligands were incubated at 37 °C with different amounts of HCMV in buffer III (20 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$) for 15 min. The mixtures were then passed through a Millipore membrane of 50 nm pore size that had been preequilibrated with buffer III. After an extensive wash, the membranes were soaked under denaturing conditions in buffer IV (10 M urea, 20 mM Tris, pH 7.5, 5 mM EDTA) to allow the RNA ligands to be released from the bound complexes. RNA ligands were finally purified by phenol/chloroform extraction followed by ethanol precipitation and were used as templates for reverse transcription in the presence of 7.5 $\mu$M of primer JH1052 and avian myoblastosis virus (AMV) reverse transcriptase (Promega Inc., Madison, Wisconsin) (Liu & Altman, 1994). The synthesized cDNA was subsequently amplified by PCR with oligodeoxynucleotide primers JH1031 and JH1052 and was used to generate RNA ligands for the next round of selection. PCR was carried out in a reaction mixture (100 $\mu$L) that contained 10 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 0.01% gelatin, 50 mM KCl, 0.2 mM dNTPs, 1 U Taq DNA polymerase (Perkin-Elmer) and 100 pmol each of oligodeoxynucleotides JH1031 and JH1052 (Liu & Altman, 1994).

Initially, 2 nmol of the pool of RNA ligands that contained the randomized sequence were mixed with $1 \times 10^9$ pfu of HCMV in a volume of 5 mL. In subsequent cycles of selection, 10 pmol of RNA ligands were used and mixed with HCMV in a volume of 500 $\mu$L. During the first four cycles of selection, viral particles of 5–10 $\times$ $10^6$ pfu were used. During the selection cycles 5–8, 9–12, and 13–16, the amounts of HCMV used were reduced to $1 \times 10^5$, $5 \times 10^3$, and $5 \times 10^2$ pfu, respectively. The affinity of the RNA ligand population after every other cycle of selection was assayed to monitor the selection progress. After 16 cycles of selection, cDNA that contained ligand sequences was cloned into pUC19 and sequence analysis was performed with Sequenase in the presence of $\alpha$-[$^{35}$S]-ATP (Amersham).

## In vitro assays for binding of selected RNA analogs to HCMV particles

Binding assays were carried out by mixing 5 $\mu$L of different concentrations of HCMV to 45 $\mu$L of either binding buffer III or culture medium DMEM that contained 10,000–100,000 cpm of RNA ligands (less than 100 fmol). The mixture was incubated at 37 °C for 15 min and then passed over a 50 nm pore size filter, which was either preequilibrated in buffer III or culture medium DMEM. After extensive washing, the filter was dried and the radioactivity was quantitated with a STORM840 PhosphorImager. Each binding curve consisted of at least six points and each point represented the averages of values obtained from at least two independent experiments.

## Viral infection and assays of the antiviral activity of the ligands

A well (of a 6- or 24-well dish) of cells (approximately 1–5 $\times$ $10^5$ cells) were either mock infected or infected with HCMV at a MOI of 0.001–0.01 (for plaque assay) or 0.2–1 (for viral yield assays) in an inoculum of 200 $\mu$L (for 24-well plates) or 500 $\mu$L (for 6-well plates). The viruses were preincubated at 37 °C for 15 min with DMEM alone or DMEM that contained different concentrations of L13, L17, L19, $G_0$, or nAb, a mixture of anti-HCMV gB neutralizing antibodies. After a 2-h exposure to the virus at 37 °C, the cells were extensively washed to remove unbound viruses and then incubated with DMEM supplemented with 10% fetal bovine serum. The infected cells were incubated for a certain period time (as stated in Results) for plaque assays or before being harvested for titration of viral yields. To determine the viral yields, viral stocks were prepared by sonicating the infected cell lysates, and their titers were determined by infecting $1 \times 10^5$ HFF cells in 24-well plates and counting the number of plaques 10–14 days postinfection.

## Isolation of protein extracts, electrophoretic separation, and staining of infected cellular polypeptides with antibodies

To prepare protein extracts, cells were harvested, washed, and lysed as described previously (Liu & Altman, 1995). The proteins were separated on 9% [v/v] SDS-polyacrylamide gels crosslinked with *N,N″*-methylenebisacylamide, transferred electrically to nitrocellulose membranes, and stained with anti-mouse IgG conjugated with horseradish peroxidase and the mAb1203 antibody against HCMV IE72 (Liu & Altman, 1995). To quantify the expression levels of viral IE72, the membranes were stained with a chemiluminescent substrate (Amersham) and subsequently scanned in a STORM840 PhosphorImager (Molecular Dynamics, Sunnyvale, California).

### Assays of number of cells expressing IE72 and the intracellular level of viral DNA

Immunofluorescent staining of cells that expressed IE72 was used to determine the number of the infected cells as described previously (Navarro et al., 1993). In brief, $10^5$ cells grown on 10-mm round coverslips were infected with viruses that were pretreated with DMEM containing different concentrations of L19 or $G_0$. After a 1.5-h incubation at 37 °C, cells were extensively washed and then incubated with fresh media containing 10% fetal bovine serum. At 36 h after infection, cells were fixed with an acetone and methanol mixture (1:1, v/v) for 20 min and washed three times in phosphate-buffered saline (PBS). The fixed cells were incubated with anti-IE72 monoclonal antibody, mAb1203 (diluted 1:500 in PBS), for 30 min, and then rinsed three times in PBS, followed by a 30-min incubation with anti-mouse IgG-fluorescein isothiocyanate antibody (Vector Labs, Burlingame, California) diluted 1:250 in PBS. The stained cell nuclei were visualized by fluorescence microscopy and their numbers were counted. At least 10 microscopic fields (40×), corresponding to at least 3,000 cells, were scored for specific nuclear staining and the average of the numbers of the staining cells was calculated.

Viral DNA was detected by PCR amplification of the viral immediate-early IE1/IE2 sequence. The 5′ and 3′ primers were CMV3 (5′-CCAAGCGGCCTCTGATAACCAAGCC-3′) and CMV4 (5′-CAGCACCATCCTCCTCTTCCTCTGG-3′), respectively. PCR cycles and other conditions were optimized to assure that the amplification was within the linear range. To obtain the PCR DNA template, $5 \times 10^5$ cells grown on 6-well plates were infected with viruses that were pretreated with DMEM alone or DMEM that contained 200 $\mu$g/mL anti-HCMV gB neutralizing antibodies, 50 $\mu$g/mL heparin, or different concentrations of L19 and $G_0$. After a 1.5-h incubation at 37 °C, the inoculum was removed and cells were washed three times with PBS. Then the cells were treated with 0.25% trypsin and harvested. The harvested cells were further treated with 200 $\mu$g/mL trypsin at 37 °C for 10 min and washed with PBS. Under these conditions, most of the HCMV surface glycoproteins were digested and were not detectable (Baldick & Shenk, 1996). The trypsin treatment and wash with PBS was repeated three times to completely remove the unbound viruses and those that still attached to the cells. The cell pellet was resuspended in the lysis buffer (50 mM Tris, pH 9.0, 1 mM EDTA, 100 mM NaCl) containing 500 $\mu$g/mL Proteinase K and 1.0% SDS and incubated at 55 °C for 2 h. The digested mixture was extracted with phenol/chloroform three times and chloroform once followed by ethanol precipitation to generate the PCR DNA templates. The PCR reaction consisted of 20 cycles with denaturation at 94 °C for 1 min, followed by primer annealing at 47 °C for 1 min, and extension at 72 °C for 1 min. The last cycle was again an extension at 72 °C for 10 min. Human $\beta$-actin sequence was used as the internal control. The 5′ and 3′ primers used to amplify the actin sequence were Actin5 (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′) and Actin3 (5′-CTAGAAGCATTGCGGTGGCAGATGGAGGG-3′), respectively (Stratagene Inc., La Jolla, California). The amplified HCMV DNA (481 bp) and actin sequence (610 bp) were separated on either 1% agarose gels or 4% nondenaturing polyacrylamide gels.

To quantitate the level of viral DNA, PCR reactions were carried out in the presence of $\alpha$-[$^{32}$P]-dCTP. The radiolabeled DNA samples separated on either agarose or polyacrylamide gels were scanned with a STORM840 PhosphorImager. A standard (dilution) curve was generated by amplifying different dilutions of the template DNA. The plot of counts for both HCMV and $\beta$-actin versus dilutions of DNA did not reach a plateau for the saturation curve (data not shown) under the conditions described above, indicating that quantitation of viral DNA could be accomplished. The fact that the ratio of viral DNA to $\beta$-actin remained constant with respect to each DNA dilution in the standard curve indicated the adequate accuracy and reproducibility of the assay. The PCR results were derived from three independent experiments.

### UV-crosslinking experiments

UV crosslinking between the uniformly [$^{32}$P]-labeled ligands and HCMV was carried out essentially as described by Guerrier-Takada et al. (1989). To prevent nonspecific binding of the ligands to HCMV, HCMV was mixed with the radiolabeled ligand in the presence of a 20-fold molar excess of $tRNA^{Tyr}$ molecules. Binding of L19 and L13 to HCMV was not affected in the presence of $tRNA^{Tyr}$ (Fig. 2B) and binding of $tRNA^{Tyr}$ to HCMV did not reduce the infectivity of HCMV particles (data not shown). In the competition experiments with $G_0$, L13, or L19, these nonradiolabeled ligand molecules were included in the mixture and were in 20-fold molar excess to the radiolabeled ligands. The radiolabeled RNA ligands (5–10 nM) as well as $tRNA^{Tyr}$ and nonradiolabeled ligand molecules were incubated with HCMV virion ($1 \times 10^4$ pfu) in binding buffer III for 15 min at 37 °C. Then, the reaction mixture was exposed to UV light (254 nm) for 5–15 min and then digested with 40 $\mu$g/mL RNase A, 60 U/mL RNase T1, and 400 U/mL S1 nuclease for 30 min. The crosslinked mixtures were either immunoprecipitated first with monoclonal antibodies against HCMV-gB or gH or directly denatured in the disruption buffer (0.05 M Tris, pH 7.0, 8.5% [v/v] sucrose, 5% [v/v] $\beta$-mercaptoethanol, 2% [v/v] sodium dodecyl sulphate). The immunoprecipitation was carried out as described previously (Pereira et al., 1982; Meyer et al., 1990). The protein samples were boiled for 1 min, then subjected to electrophoretic separation in 7.5% [v/v] SDS-polyacrylamide denaturing gels, and finally analyzed by a STORM840 PhosphorImager.

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