Tohoku J. Exp. Med., 2000, **192**, 141-149

Herpesvirus Alkaline Deoxyribonuclease; a Possible Candidate as a Novel Target for Anti-Herpesvirus Therapy

Atsushi Chiba, Masahiro Ogasawara, Itsuro Yoshida, Yoko M. Knox and Tatsuo Suzutani

Department of Microbiology, Asahikawa Medical College, Asahikawa 078-8510

Chiba, A., Ogasawara, M., Yoshida, I., Knox, Y.M. and Suzutani, T. *Herpesvirus Alkaline Deoxyribonuclease; a Possible Candidate as a Novel Target for Anti-Herpesvirus Therapy*. Tohoku J. Exp. Med., 2000, **192** (2), 141-149 —— Herpesvirus alkaline deoxyribonucrease (DNase) is coded in the genome of all herpesvirus species determined total sequence and is conserved in structure. In order to determine whether the enzyme could be a target for a novel anti-herpesvirus therapy, the anti-herpes simplex virus type 1 (HSV-1) activity of antisense oligonucleotide for HSV-1 alkaline DNase was studied. Six antisense phosphorothioate oligonucleotides, targeted to an internal AUG start codon, were designed and evaluated. One of the oligonucleotides, UL12-4, inhibited wild type and thymidine kinase-deficient HSV-1 replication to 21.5 and 19.5% at 40 μM, respectively. The quantity of alkaline DNase mRNA and DNase activity in HSV-1-infected Vero cells was reduced to one eighth and 66.9% of control, respectively, by treatment with 40 μM of UL12-4, but no effect was observed on the quantity of HSV-1 glycoprotein H mRNA (γ_2 gene) or on the replication of Vero cells. These results indicate that UL12-4 inhibits HSV-1 replication by decreasing the amount of alkaline DNase mRNA. The herpesvirus alkaline DNase could be a novel target for anti-herpesvirus drug. ———— herpes simplex virus; alkaline DNase; antisense oligonucleotide

During the last decade, acyclovir has been used widely for the treatment of herpesvirus infection, resulting in a significant reduction in the mortality and length of the disease's course in patients with herpes simplex virus (HSV) and varicella-zoster virus (VZV) infection. However, as the number of patients treated with acyclovir has increased, an increasing number of acyclovir-resistant virus strains, mainly from immunocompromised patients, have been isolated (Burns et al. 1982; Wade et al. 1983; Chatis and Crumpacker 1992). The majority of these are thymidine kinase (TK) mutants which lose their TK activity or affinity for acyclovir. The present choice of treatment for patients with an

Received August 21, 2000; revision accepted for publication October 15, 2000.

Address for reprints: Tatsuo Suzutani, Department of Microbiology, Asahikawa Medical College, 2-1-1-1 Midorigaokahigashi, Asahikawa 078-8510, Japan.
e-mail: suzutani@asahikawa-med.ac.jp

acyclovir-resistant virus infection is only vidarabine (9-β-D-arabinofuranosyladenine) or foscarnet (phosphonoformic acid). The development of other anti-herpes drugs, which have different mechanisms that suppress HSV and VZV replication, should continue.

Alkaline deoxyribonuclease (DNase), coded by the UL12 gene of HSV, is a relatively abundant phospholylated protein found in the intranuclear lesion of an infected cell as a complex with the major DNA binding protein (UL29) (Heir and Gold 1963; Hoffman and Cheng 1978; Vaughan et al. 1984; De Bruyn Kops and Knipe 1988; Thomas et al. 1992; Daikoku et al. 1995). The precise role of this enzyme has not been clarified, though Martinez et al. (1996a) reported that DNA is more branched in alkaline DNase-deficient HSV infected cells than in wild type virus infected cells. Alkaline DNase may play an important role in the viral life cycle and it may be a possible target for wide spectrum anti-herpesvirus therapy, because the yield of DNase-deficient herpes simplex virus type 1 (HSV-1) is 10^2 to 10^3 times lower than that of the wild type (Weller et al. 1990), and alkaline DNase is present in all herpesvirus species (Martinez et al. 1996b). In this study, we analyzed whether the inhibition of DNase activity causes a reduction in viral replication using antisense oligonucleotides.

Materials and Methods

Cells and viruses

Vero cells were obtained from Flow Laboratories Inc. (McLean, VA, USA). The cell lines were cultured in MEM supplemented with 10% (vol/vol) newborn calf serum. The VR-3 strain of HSV-1 was supplied by the American Type Culture Collection (Rockville, MD, USA). The TK-deficient mutant of the VR-3 strain (the VRTK$^-$ strain) was isolated in our laboratory (Suzutani et al. 1988, 1995).

Oligonucleotides

Phosphorothioate oligonucleotides were used as antisense oligonucleotides in this study. The oligonucleotides were synthesized by Hokkaido System Science Co., Ltd. (Sapporo) using a DNA synthesizer Expedite (PerSeptive Biosystems, Framingham, MA, USA) and purified using a HPLC and TSKgel OligoDNA RP 4.6 mm × 15 cm column (Tosoh, Tokyo). The concentration of the oligonucleotide stock solutions was calculated at an optical density of 260 nm.

Yield reduction assay

The antiviral activities of the oligonucleotides were evaluated by a yield reduction assay using Vero cells. Confluent cells in 35 mm dishes were infected with HSV-1 at 0.1 plaque forming unit (pfu) per cell and were incubated with MEM containing 2% newborn calf serum and various amounts of antisense oligonucleotides for 48 hours in a CO_2 incubator. The cultures were frozen and

thawed three times and the cellular debris was removed by centrifugation at 1000×g for 10 minutes at 4°C. The supernatants were stored at −80°C until titration. The titers of HSV in the samples were determined by a plaque assay on the Vero cells.

Inhibitory effects of the oligonucleotides on cell growth

The 50% inhibitory concentration (IC_{50}) of the oligonucleotides for Vero cell growth was determined as described previously (Koyano et al. 1996).

Semi-quantitative RT-PCR

The quantity of the mRNA of alkaline DNase and glycoprotein H in HSV-1-infected cells was determined by reverse transcription polymerase chain reaction (RT-PCR). Vero cells in 35 mm petri dishes were infected with the VR-3 strain at 2 pfu per cell and cultured in MEM-2% calf serum supplemented with 0, 10, or 40 μM of antisense oligonucleotide UL12-4 (Table 1). After an 8 hour-incubation, the mRNA was extracted from the infected cells using a mRNA isolation kit (Micro-Fast Track; Invitrogen Co., Carlsbad, CA, USA), and the first strand cDNA was synthesized using a T-Primed First-Strand Kit (Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK). The cDNA samples were serially diluted with water, and then the cDNA of alkaline DNase or of glycoprotein H in each of the diluted samples was amplified by PCR with the oligonucleotides K-up1 and K-R3, or gH-end and gH-Kpn as primers. The sequence of the primers was 5′-ACAAGGCGATACTGTCGTCG-3′ for K-up1 (nucleotide 27 057 to 27 037), 5′-GTGCTTCAGCCACTGGGCG-3′ for K-R3 (nucleotide 26 109 to 26 129), 5′-TTCGCGTCTCCAAAAAAACGG-3′ for gH-end (nucleotide 43 869 to 43 890), and 5′-CAGTCTGCCAACGTCGAGCC-3′ (nucleotide 44 420 to 44 401)

TABLE 1. *Inhibitory effects of antisense oligonucleotides against the wild type and TK deficient HSV-1, and Vero cell replication*

Antisense	Sequence	Virus titer (% control)		IC_{50} (μM)
		VR-3	VRTK⁻	Vero
UL12-1	5′-CC TAC CGT GGA CTC CAT TTC	69.2±10.5	27.2±10.6*	230
UL12-2	5′-AC CGT GGA CTC CAT TTC CGA	63.1± 8.7	34.1± 8.9*	>250
UL12-3	5′-GT GGA CTC CAT TTC CGA GAC	56.9± 5.3	NT	NT
UL12-4	5′-GA CTC CAT TTC CGA GAC GAC	21.5± 6.1*	19.5± 7.8*	>250
UL12-5	5′-TC CAT TTC CGA GAC GAC GTG	66.7± 7.6	40.9± 4.2	>250
UL12-4/neg	5′-GA CTC acT TTC Cag GAC GAC	105 ± 9.8	97.1± 9.5	>250

Sequences of the antisense oligonucleotides for the coding region are underlined. Lowercase letters indicate mismatched nucleotides. Inhibition (%) indicates virus titer at 40 μM antisense oligonucleotides as compared to untreated control. IC_{50} indicates 50% inhibitory concentration for cell growth. NT, not tested.

*$p<0.05$ compared to the untreated control.

for gH-Kpn (McGeoch et al. 1988). Thirty cycles of denaturation (94°C, 30 seconds), annealing (55°C, 1 minute), and extension (72°C, 2 minutes) following the initial denaturation at 94°C for 1 minute was performed for the PCR. The amount of PCR product was analyzed by electrophoresis on a 1% agarose gel.

Quantitative analysis of DNA synthesis

The effect of antisense oligonucleotide on virus DNA synthesis was evaluated by the incorporation of [^{3}H-methyl]thymidine. Confluent Vero cell monolayers in 35 mm dishes were cultured in MEM-0.2% calf serum for 24 hours to decrease the cellular DNA synthesis. The cultures were infected with the VR-3 strain at 2 pfu per cell and incubated in MEM-2% calf serum supplemented with 1 μCi/ml of [^{3}H-methyl]thymidine, and 0–40 μM of oligonucleotide or 0–40 μg/ml of acyclovir for 8 hours. DNA was extracted from the infected cells with proteinase K-treatment, ribonuclease-treatment, phenol/chloroform extract, trichloroacetic acid precipitation, and ethanol precipitation. The amount of the incorporated [^{3}H-methyl]thymidine was measured in a liquid scintillation counter.

Alkaline DNase assay

The alkaline DNase assay was performed as described previously with some modification (Francke et al. 1978). Confluent Vero cells were infected with the VR-3 strain at 2 pfu per cell and incubated in medium containing various concentrations of antisense oligonucleotides or acyclovir for the desired periods. The cells were harvested by trypsinization, washed with PBS, suspended in DNase buffer (50 mM Tris-HCl pH 9.0, 2 mM $MgCl_2$, and 10 mM 2-mercaptoethanol), and disrupted by sonication. After centrifugation at 12 000 rpm for 10 minutes using a micro-centrifuge, the supernatant was used as crude enzyme material. The assay mixture contained the crude enzyme material and ^{3}H-labeled *E. coli* DNA in the DNase buffer. The degradation of the labeled DNA was monitored by precipitating the undigested DNA with 10% trichloroacetic acid and counting the acid-soluble nucleotides using a scintillation counter.

Results

The inhibitory effect of antisense oligonucleotides against HSV-1 replication was evaluated by a yield reduction assay and the results are shown in Table 1. UL12–4 had the most potent activity among antisense oligonucleotides against both the wild type and TK-deficient HSV-1 strains and it had no cell toxic activity. UL12–5 and UL12–3 which hybridize three nucleotides upstream or downstream of the mRNA, had weak anti-HSV-1 activity. Each oligonucleotide could inhibit the TK-deficient HSV-1 strain more effectively than the wild type HSV-1. UL12–4/neg, which was designed as a mismatch control of UL12–4, did not show anti-HSV-1 and cell toxic activity.

To clarify the mechanism of the inhibitory effect of the antisense oligonu-

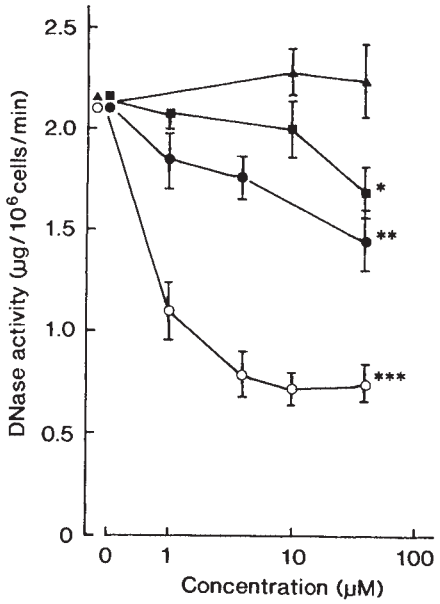

Fig. 1. DNase activity in HSV-1 infected cells. A) HSV-1-infected Vero cells were incubated with various concentrations of antisense UL12-4 (●), UL12-5 (■), UL12-4/neg (▲), or acyclovir (○) for indicated time. DNase activity in these cells was determined in the alkaline condition as described in the materials and methods. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ compared to the control.

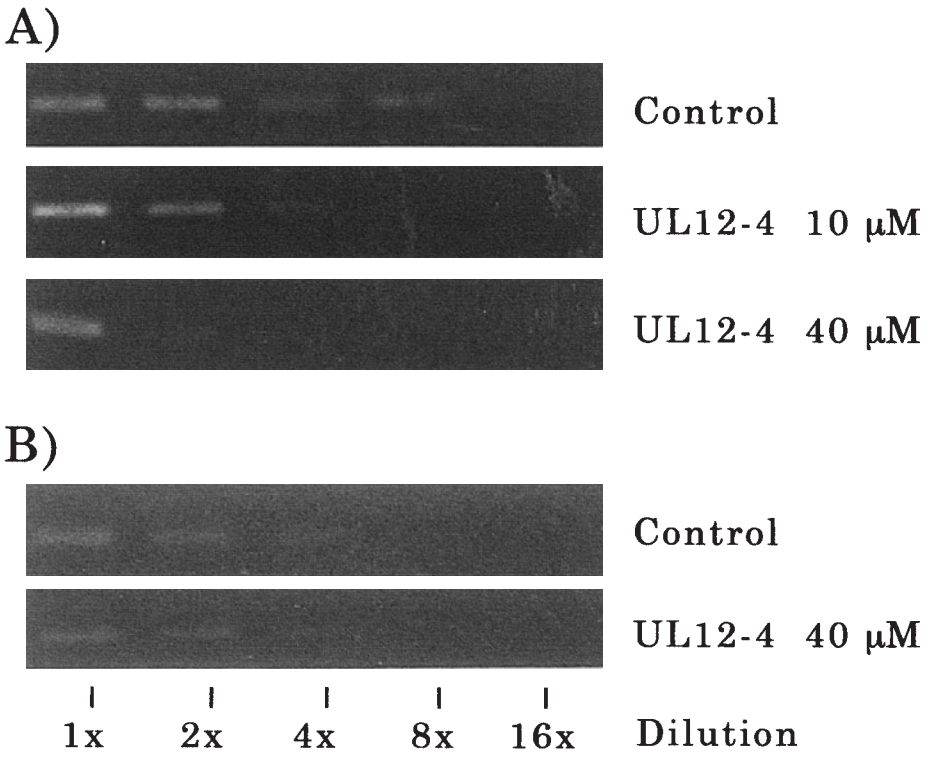

Fig. 2. Quantitative RT-PCR analysis of HSV mRNA. The effect of antisense UL12-4 on the amount of alkaline DNase mRNA (A) and glycoprotein H mRNA (B) in Vero cells at 8 hours post HSV-1-infection, were analyzed with RT-PCR. cDNA samples prepared from 2.4×10^5 cells were suspended in 50 μl of water. After the serially dilution of the cDNA samples, the quantity of mRNA in 1 μl of each of the diluted samples was analyzed.

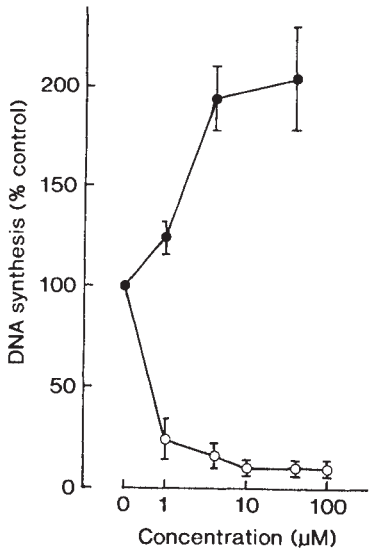

Fig. 3. Effect of antisense UL12-4 and acyclovir on DNA synthesis in HSV-1-infected Vero cells. The amount of DNA synthesis was evaluated by the uptake of [^{3}H-methyl]thymidine for 8 hours after HSV-infection and it is indicated as a percentage compared to the control HSV-1-infected cell (●, UL12-4; ○, acyclovir).

cleotides against HSV-1 replication, the effects of antisense on alkaline DNase activity, amount of mRNA of DNase and glycoprotein H, and DNA replication were examined. UL12-4 and UL12-5 inhibited DNase activity in a dose-dependent manner, but UL12-4/neg did not. Certainly, the inhibited DNase activity by antisense oligonucleotides was that of viral enzyme, because low cellular DNase activity (7% of viral DNase activity) was detected in the alkaline assay condition. The DNase activity, when 40 μM of antisense oligonucleotide was added, was reduced to 66.9 and 75.6% of control, respectively (Fig. 1). The amount of the mRNA of DNase in HSV-1 infected Vero cells decreased to about one forth or one eighth, when 10 or 40 μM of UL12-4 was added, respectively (Fig. 2A). No effect on the amount of mRNA of glycoprotein H (γ_2 gene) was observed (Fig. 2B). This result indicated that the effect of the antisense oligonucleotide against the mRNA of the DNase was specific. DNA synthesis in HSV-1 infected Vero cells was increased by antisense treatment (Fig. 3).

As a control experiment, the effect of acyclovir on DNase activity and DNA synthesis was observed. Acyclovir had a significant inhibitory effect on DNA synthesis (Fig. 3) and it suppressed DNase activity (Fig. 1).

Discussion

A series of antisense phosphorothioate oligonucleotides, targeted to the internal AUG start codon of HSV-1 alkaline DNase mRNA, were designed and tested to estimate a possibility of alkaline DNase as a novel target for anti-herpesvirus chemotherapy. UL12-4 was the most effective and it inhibited the replication of the wild type and the TK-deficient HSV-1 strains to 21.5 and 19.5% of control at

40 μM, respectively. The antiviral activity of UL12-4 was less than that of the antisense oligonucleotide which targeted the start codon of protein kinase (UL13), but it was comparable to the antisense oligonucleotides that targeted the coding region of DNA polymerase (UL30), dsDNA binding protein (UL42) and primase/helicase (UL52), the start codon of VP16 (UL48) and IE110, and the splicing site of IE4 (Kulka et al. 1989; Hoke et al. 1991; Peyman and Uhlmann 1996). The yield of protein kinase (UL13)-deficient HSV-1 was 10 fold less than that of the wild type strain and antisense to protein kinase inhibited HSV-1 replication to 9% at 4 μM (Hoke et al. 1991; Coulter et al. 1993; Overton et al. 1994). If the anti-HSV activity of the antisense oligonucleotide was caused by a specific inhibitory effect on protein kinase, the antisense might inhibit the expression of protein kinase activity completely. On the other hand, 40 μM of antisense UL12-4 inhibited the alkaline DNase activity by 66.9% of the untreated control. The insignificant effect of antisense on alkaline DNase may be caused by the composition of alkaline DNase, which is an unusual enzyme in a abundant protein in HSV-infected cells (Banks et al. 1983).

The mechanism of the anti-HSV activity of the antisense oligonucleotides was partially demonstrated. The antisense oligonucleotides decreased the amount of DNase mRNA, leading to a low expression of DNase activity and a decreased viral yield. This effect was specific because the antisense did not inhibit viral DNA synthesis, and it did not have any non specific activity such as cell toxicity or suppression of glycoprotein H mRNA. Moreover, the negative control oligonucleotide, UL12-4/neg, did not have effect on virus replication and DNase activity. The antisense oligonucleotides designed and used in this study could be inhibiting the gene expression at the level of mRNA processing or by mRNA degradation by RNase H at the site of the RNA-DNA heteroduplex (Walder and Walder 1988).

As a control experiment, we analyzed the effect of acyclovir on alkaline DNase activity (Fig. 1). Surprisingly, acyclovir had the most potent inhibitory effect on the expression of DNase activity. If this effect was caused by the inhibition of the viral DNA synthesis, then the alkaline DNase gene might be an early-late gene (γ_1 gene) although a previous report indicated early gene (Costa et al. 1983). Another possibility was that the DNase activity could have been inhibited following the inhibition of the DNA binding protein activity by acyclovir, considering a previous report that describes the DNA binding protein influence on alkaline DNase activity (Littler et al. 1983). Further study is necessary for an accurate explanation of how ACV influences alkaline DNase activity.

One of objectives of anti-herpesvirus therapy is to develop a novel drug which affects all species of herpesviridae. Alkaline DNase which is conserved in all herpes species in our present knowledge was chosen and studied as a novel target. We showed that the antisense oligonucleotides targeted to the translation initia-

tion site of alkaline DNase mRNA diminished the amount of mRNA which lead to a decrease in DNase activity and virus yield. However, the amino-terminal site of HSV-1 DNase are not present in other herpesvirus DNases (Martinez et al. 1996b). Further studies should be done to develop antisense nucleotides which target the homologous segment of herpesvirus alkaline DNase genes.

References

1) Banks, L., Purifoy, D.J.M., Hurst, P.-F., Killington, R.A. & Powell, K.L. (1983) Herpes simplex virus non-structural proteins. IV. Purification of the virus-induced deoxyribonuclease and characterization of the enzyme using monoclonal antibodies. *J. Gen. Virol.*, **64**, 2249–2260.
2) Burns, W.H., Sanat, R. & Santos, G.W. (1982) Isolation and characterization of resistant herpes simplex virus after acyclovir therapy. *Lancet*, **I**, 421–424.
3) Chatis, P.A. & Crumpacker, C.S. (1992) Resistance of herpesviruses to antiviral drugs. *Antimicrob. Agents Chemother.*, **36**, 1589–1595.
4) Costa, R.H., Draper, K.G., Banks, L., Powell, K.L., Cohen, G., Eisenberg, R. & Wagner, E.K. (1983) High-resolution characterization of herpes simplex virus type 1 transcripts encoding alkaline exonuclease 50 000-dalton protein tentatively identified as a capsid protein. *J. Virol.*, **48**, 591–603.
5) Coulter, L.J., Moss, H.W., Lang, J. & McGeoch, D.J. (1993) A mutant of herpes simplex virus type 1 in which the UL13 protein kinase gene is disrupted. *J. Gen. Virol.*, **74**, 387–395.
6) Daikoku, T., Yamashita, Y., Tsurumi, T. & Nishiyama, Y. (1995) The US3 protein kinase of herpes simplex virus type 2 is associated with phosphorylation of the UL12 alkaline nuclease in vitro. *Arch. Virol.*, **140**, 1637–1644.
7) De Bruyn Kops, A. & Knipe, D.M. (1988) Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. *Cell*, **55**, 933–941.
8) Francke, B., Moss, H., Timbury, M.C. & Hay, J. (1978) Alkaline DNase activity in cells infected with a temperature-sensitive mutant of herpes simplex virus type 2. *J. Virol.*, **26**, 209–213.
9) Heir, H.M. & Gold, E. (1963) Deoxyribonucleic acid nucleotidyltransferase and deoxyribonuclease from cultured cells infected with herpes simplex virus. *Biochim. Biophys. Acta*, **72**, 263–276.
10) Hoffman, P.J. & Cheng, Y.-C. (1978) The deoxyribonuclease induced after infection of KB cells by herpes simplex virus type 1 or type 2. *J. Biol. Chem.*, **253**, 3557–3562.
11) Hoke, G.D., Draper, K., Freier, S.M., Gonzalez, C., Driver, V.B., Zounes, M.C. & Ecker, D.J. (1991) Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection. *Nucleic. Acid Res.*, **19**, 5743–5748.
12) Koyano, S., Suzutani, T., Yoshida, I. & Azuma, M. (1996) Analysis of phosphorylation pathways of antiherpesvirus nucleosides by varicella-zoster virus-specific enzymes. *Antimicrob. Agents Chemother.*, **40**, 920–923.
13) Kulka, M., Smith, C.C., Aurelian, L., Fishelevich, R., Meade, K., Miller, P. & Ts'o, P.O. (1989) Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonate)s complementary to the acceptor splice junction of herpes simplex virus type 1 immediate early mRNA 4. *Proc. Natl. Acad. Sci. USA*, **86**, 6868–6872.
14) Littler, E., Purifoy, D.J.M., Minson, A. & Powell, K.L. (1983) Herpes simplex virus non-structural proteins. III. Function of the major DNA-binding protein. *J. Gen. Virol.*, **64**, 983–995.
15) Martinez, R., Sarisky, R.T., Weber, P.C. & Weller, S.K. (1996a) Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication

intermediates. *J. Virol.* **70**, 2075–2085.

16) Martinez, R., Shao, L., Bronstein, J.C., Weber, P.C. & Weller, S.K. (1996b) The product of a 1.9-kb mRNA which overlaps the HSV-1 alkaline nuclease gene (UL12) cannot relieve the growth defects of a null mutant. *Virology*, **215**, 152–164.

17) McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. & Taylor, P. (1988) The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.*, **69**, 1531–1574.

18) Overton, H., McMillan, D., Hope, L. & Wong-Kai-In, P. (1994) Production of host shutoff-defective mutants of herpes simplex virus type 1 by inactivation of the UL13 gene. *Virology*, **202**, 97–106.

19) Peyman, A. & Uhlmann, E. (1996) Minimally modified oligonucleotides-combination of end-capping and pyrimidine-protection. *Biol. Chem. Hoppe-Seyler*, **377**, 67–70.

20) Suzutani, T., Machida, H., Sakuma, T. & Azuma, M. (1988) Effects of various nucleosides on antiviral activity and metabolism of 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil against herpes simplex virus types 1 and 2. *Antimicrob. Agents Chemother.*, **32**, 1547–1551.

21) Suzutani, T., Koyano, S., Takada, M., Yoshida, I. & Azuma, M. (1995) Analysis of the relationship between cellular thymidine kinase activity and virulence of thymidine kinase-negative herpes simplex virus types 1 and 2. *Microbiol. Immunol.*, **39**, 787–794.

22) Thomas, M.S., Gao, M., Knipe, D.M. & Powell, K.L. (1992) Association between the herpes simplex virus major DNA-binding protein and alkaline nuclease. *J. Virol.*, **66**, 1152–1161.

23) Vaughan, P.J., Banks, L.M., Purifoy, D.J.M. & Powell, K.L. (1984) Interactions between herpes simplex virus DNA-binding proteins. *J. Gen. Virol.*, **65**, 2033–2041.

24) Wade, J.C., Mclaren, C. & Meyer, J.D. (1983) Frequency and significance of acyclovir-resistant herpes simplex virus isolated from marrow transplant patients receiving multiple courses of treatment with acyclovir. *J. Infect. Dis.*, **148**, 1077–1082.

25) Walder, R.Y. & Walder, J.A. (1988) Role of RNase in hybrid-arrested translation by antisens oligonucleotides. *Proc. Natl. Acad. Sci. USA*, **85**, 5011–5015.

26) Weller, S.K., Seghatoleslami, M.R., Shao, L., Rowse, D. & Carmichael, E.P. (1990) The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant. *J. Gen. Virol.*, **71**, 2941–2952.