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Induction of deoxycytidine deaminase activity in mammalian cell lines by infection with herpes simplex virus type 1

(mouse mutant cell lines/selective system/thymidine kinase/antiviral chemotherapy)

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ABSTRACT **Herpes simplex virus type 1 induces deoxycytidine deaminase (cytidine/deoxycytidine aminohydrolase, EC 3.5.4.5) activity when it lytically infects a number of mammalian cell lines. The deaminase activity is induced in a mouse cell line that is deficient in this enzyme. The induction of the enzyme in this mutant cell line does not occur in the presence of actinomycin D and the induced enzyme is more thermolabile than the enzyme of the wild-type mouse cell line. Furthermore, a new deoxycytidine deaminase species with a characteristic electrophoretic mobility that is different from that of the host cell enzyme is found in cell extracts prepared from a human cell line infected with herpesvirus. These results strongly suggest that the virus-induced deoxycytidine deaminase is coded by the viral genome. Because a deficiency in this enzyme is conditionally lethal for cells growing in a medium containing 5-methyldeoxycytidine as the sole source of thymidylate, this enzyme can be utilized as a selective marker for selecting mutant cells that have regained deoxycytidine deaminase activity as the result of infection by ultraviolet-inactivated herpes simplex virus.**

Herpes simplex virus (HSV) has been shown to be able to donate its gene(s) to mammalian cells under nonpermissive conditions and, as a result, alter the biochemical phenotype of the recipient cells (1, 2). The biochemical marker that has been most extensively used for such studies is thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) (TK). Activity of this enzyme is elevated in cells after lytic infection by HSV (3). There is accumulating evidence which suggests that the structural gene for the virus-induced TK is carried by the viral genome: the induced TK has kinetic properties that are different from those of the host enzyme (4), and its activity is inhibited by antiserum to virus-specific proteins (5). Moreover, mutant strains of HSV that are deficient in TK-inducing ability have been isolated (6, 7). Some of these mutants produce fragments of TK, which strongly suggest that they contain a chain-terminating mutation within the structural gene for the enzyme (7). When a TK-deficient mouse cell line was infected with UV-inactivated HSV and the infected cells were incubated in the hypoxanthine/aminopterin/thymidine selective medium (8), cells that permanently acquired TK activity were selected (1, 9). The TK in such biochemically transformed cells has the properties of the virus-induced enzyme (10). These cells contain as much as 24% of the herpes viral DNA sequences (11, 12). In order to further study this interesting viral gene transfer system, it is highly desirable to have additional selective markers that are carried by the HSV.

It has been shown that, beside TK, activities of a number of other enzymes related to DNA synthesis are induced by HSV (see ref. 13). In this paper, evidence will be presented to show that HSV type 1 (HSV-1) induces deoxycytidine deaminase (cytidine/deoxycytidine aminohydrolase, EC 3.5.4.5) (dCD) activity when it lytically infects dCD$^-$ mouse cells. It will also be shown that the induced enzyme is very likely coded by the viral genome. We have previously shown that dCD is a useful selective marker for somatic cell hybridization (14). Thus, this enzyme can also be used as a selective marker for isolating dCD$^+$ cells from dCD$^-$ cells after infection by UV-inactivated HSV-1 virus.

Abbreviations: HSV, herpes simplex virus; TK, thymidine kinase; dCD, deoxycytidine deaminase; dCK, deoxycytidine kinase; p.i., post infection.

MATERIALS AND METHODS

Cells. Cells are maintained routinely in Dulbecco-Vogt modified Eagle's medium (Gibco) supplemented with 10% calf serum (Gibco) and equilibrated with 10% CO_2 in air.

Two mouse mutant lines were used: 3T6-BCE and LMTK$^-$. 3T6-BCE, derived from the mouse 3T6 line, is deficient in both deoxycytidine kinase (NTP:deoxycyidine 5′-phosphotransferase, EC 2.7.1.74) (dCK) and dCD activities (14). LMTK$^-$ is a TK$^-$ derivative of mouse L cells (15). 3T6-BCE cells were shown to be free of mycoplasma contamination by the [^{3}H]-uridine/[^{3}H]uracil incorporation methods (16) as well as by the [^{3}H]adenosine phosphorolysis method (17).

A baby hamster kidney cell line, BHK 21/C13 (BHK), and a human cell line, HEp-2, were obtained from the American Type Culture Collection. BHK cells have been shown to lack dCD (18).

Viruses. The two strains of HSV-1, Cl-101, the original wild-type strain of Dubbs and Kit (6), and its TK$^-$ derivative (7), TK21, are generous gifts from Wm. Summers and W. Summers.

Virus is grown routinely in Vero, a monkey kidney cell line, in a medium containing 10% fetal calf serum equilibrated with 5% CO_2. For stocks of viruses, a near confluent culture of cells is infected with virus at a low multiplicity of infection (0.001–0.01 plaque-forming unit per cell) and, after two cycles of infection, cells are harvested. The cells are broken by repeated freezing-thawing followed by sonication for 30 sec. The 500 × *g* supernatant which contains viruses is collected and divided into small aliquots and stored at −70°.

Preparation of Extracts from HSV-Infected Cells. A near confluent monolayer of cells in 100 mm dishes is infected with a high multiplicity of infection (5–10 plaque-forming units per cell) of virus in 0.5 ml of medium. After adsorption of virus for 30 min, 5 ml of fresh medium containing 10% fetal calf serum is added and the plates are then incubated at 37° in 5% CO_2. At the end of the incubation period, cells are collected by scraping, washed twice with phosphate-buffered isotonic saline, and resuspended in 50 mM Tris·HCl, pH 7.4/1 mM EDTA/0.1 mM dithiothreitol/1 μM thymidine. The cells are broken by repeated freezing-thawing and sonication for 30 sec. The lysates are centrifuged at 40,000 × *g* for 30 min and the supernatant is stored in aliquots at −70° until use.

Enzyme Assays. Deoxycytidine deaminase is assayed by one

of two methods. The routine assay method is as described by Chan *et al.* (14) and utilizes a Dowex-50 column to separate [^{14}C]deoxyuridine, the product of the reaction, from the substrate, deoxycytidine.

The second assay method involves thin-layer chromatography. The reaction mixture contains 50 mM Tris·HCl, pH 8.0/1 mM EDTA/50 μM [^{14}C]deoxycytidine, specific activity 5 μCi/μmol/and cell proteins at 50–100 μg/ml. The mixture is incubated at 37° for 30 min. The reaction is stopped by chilling and the addition of perchloric acid to a final concentration of 4%. The acid is neutralized by KOH and the salt precipitate is removed by centrifugation. The supernatant fraction is then used for two-dimensional thin-layer chromatography on cellulose plates (Brinkman MN 300). The first dimension is developed with methanol/HCl/water (80:20:1, vol/vol) and the second dimension, 1-butanol/methanol/water/NH_4OH (60:20:20:1, vol/vol). Deoxyuridine is well resolved by this procedure; however, uracil and deoxycytidine migrate together. Therefore, uracil and deoxycytidine are eluted from the thin-layer plate and then separated on a Dowex-50 column (19). Enzyme activity measured by either method agrees well and is linear with respect to the amount of cell extract added and with the time of incubation.

Thymidine kinase is assayed by the method described by Long *et al.* (20). The reaction mixture containing 10 μM [^{3}H]thymidine, specific activity 3.6 Ci/mmol/5 mM ATP/5 mM $MgCl_2$/50 mM Tris·HCl buffer, pH 8.0 is incubated with cell extracts. The ^{3}H-labeled nucleotide formed by the reaction is determined by its adsorption to discs of DE-81 DEAE-cellulose paper (Whatman). Deoxycytidine kinase is assayed by a similar method (14).

Thermosensitivity Test. Cell extracts are purified on a 1 × 22 cm Sephadex G-25 column, which is equilibrated with 50 mM Tris·HCl, pH 7.4/1 mM EDTA/0.1 mM dithiothreitol. Crude extracts (0.5 ml) are applied to the column and 1 ml of the eluate that contains the highest enzyme activity is collected. Aliquots (100 μl) of purified extracts are heated to 56° or 60°. At intervals, tubes are taken out and immediately cooled to 0°. All tubes are assayed for dCD activity together.

Actinomycin D Experiment. Cell monolayers are infected as described above. At different times after infection, actinomycin D (Calbiochem) is added to a final concentration of 0.5 μg/ml and the plates are incubated further. Cells are then harvested at different time points after actinomycin D addition and the extracts are prepared by the freezing-thawing-sonication method. RNA synthesis is measured by incorporation of [^{3}H]uridine into trichloroacetic-acid-precipitable materials that are not hydrolyzed in perchloric acid (16).

Cellogel Electrophoresis. The method for electrophoretic separation of viral and cellular deoxycytidine deaminase is described elsewhere (14).

Protein Determination. Protein concentrations were determined by the method of Lowry *et al.* (21).

RESULTS

Induction of Deoxycytidine Deaminase in Cells Infected by Herpes Simplex Virus. Fig. 1 shows the pathways of deoxycytidine reutilization in mammalian cells. Deoxycytidine is phosphorylated by dCK to dCMP (22), which is further phosphorylated to di- and triphosphates. dCMP can also be deaminated to form dUMP (23), which is the immediate precursor of dTMP in the *de novo* pathways of thymidylate synthesis. Alternatively, deamination of cytosine residues can occur at the nucleoside level (24). The product of deamination, deoxyuridine, is then phosphorylated by TK to form dUMP. There are

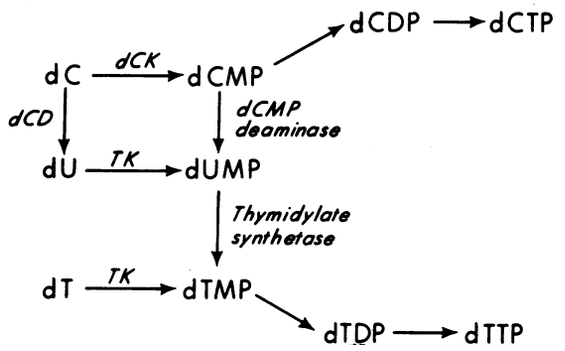

FIG. 1. Pathways of deoxycytidine reutilization in mammalian cells.

three aspects of these pathways that are relevant to present work. (*i*) Deoxycytidine could be a source of either deoxycytidine or thymidine nucleotides, depending on its state of deamination. (*ii*) dCD catalyzes the reaction one step earlier than the TK reaction in an alternate pathway of thymidylate synthesis. (*iii*) Activities of the three enzymes involved in these pathways, TK, dCK, and dCMP deaminase (EC 3.5.4.12), are known to be elevated in cells infected with HSV (see ref. 13).

A mouse cell line resistant to both cytosine arabinonucleoside and bromodeoxycytidine has been derived (14). This line was previously shown to be completely lacking in dCD (as well as dCK) activities. When these dCD-deficient cells are infected with HSV-1, they are found to contain very high levels of dCD activity. Fig. 2 shows the time course of the induction of dCD activity in dCD$^-$ cells infected with HSV-1. The dCD activity appeared within the first 3 hr after infection, increased exponentially in the next 20 hr, and then leveled off. At the maximal level of induction the specific enzyme activity in the infected cells is more than 200 times that in uninfected dCK$^-$dCD$^-$ cells and 7 to 10 times that in uninfected wild-type cells.

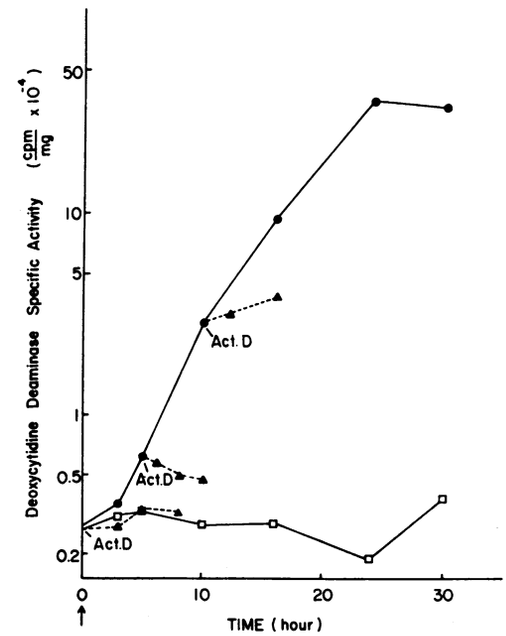

FIG. 2. Time course of induction of deoxycytidine deaminase. 3T6-BCE cells are infected with HSV-1 at time 0. Actinomycin D is added at different times after infection as indicated. Cells are then harvested at time intervals for dCD assays. Infected cells without added actinomycin D, (●—●); infected cells with added actinomycin D, (▲- - - - -▲); uninfected cells, (□—□).

Table 1. Deoxycytidine deaminase (dCD) and thymidine kinase (TK) activities in LMTK$^-$ cells and deoxycytidine deaminase activity in BHK cells, both infected with wild-type HSV-1

Hour p.i.	Specific enzyme activity*		
	LMTK$^-$		BHK
	dCD	TK	dCD
0	0.28	0.05	0.035
3	0.28	0.05	0.028
6	0.22	0.05	0.044
10	0.27	0.05	—
12	—	—	0.030
18	0.66	0.27	2.21
24	1.32	2.66	2.15
30	1.28	2.45	—

* Specific enzyme activity is expressed in nmol/min per mg of protein at 37°.

Similar experiments were done using another mouse cell line, LMTK$^-$, which is a TK$^-$ subline of L cells. The purpose of these experiments is to compare the time course of induction of both dCD and TK by assaying the infected cell extracts for both enzymes. The results, presented in Table 1, show that, in infected LMTK$^-$ cells, dCD activity is elevated at least 6 times that in uninfected cells. Because these cells are virtually devoid of TK activity, the increase in TK activity is even more dramatic (to about 60 times the activity in uninfected cells). The time course of dCD induction approximately parallels that of TK induction and they both reach a maximum at 24 hr post infection (p.i.).

It has been reported that the hamster cell line, BHK, lacks dCD (18). Therefore, this line was also examined for the induction of dCD by infection with HSV-1. The results (Table 1) show that the dCD activity increases soon after infection and reaches a maximum level about 12–16 hr p.i. In this line, the time at which the enzyme activity is maximally induced is considerably earlier than that in either 3T6-BCE or LMTK$^-$ cells infected with HSV. Because, compared to BHK, LMTK$^-$ (and probably 3T6-BCE) cells are poor host cells for herpes infection (1), this discrepancy could probably be attributed to cell strain differences.

Characterization of Reaction Product. Because of the complicated pathways for deoxycytidine reutilization in mammalian cells, experiments were undertaken to identify the reaction products of our dCD assays. The major product of the dCD assay using infected cell extracts is identified as deoxyuridine by two-dimensional thin-layer chromatography. We also eluted material that comigrated with deoxycytidine and passed it through a Dowex 50 column, in order to separate uracil from deoxycytidine. It was found by this method that uracil is another reaction product. This result is consistent with our previous report that cultured mouse cells contain a high uridine/deoxyuridine phosphorylase (EC 2.4.2.3) activity (19).

Is the Measured dCD Activity Actually the dCMP Deaminase Activity? Because deoxycytidine can be deaminated after its phosphorylation, deoxyuridine can also be generated *via* the following pathway (Fig. 1): deoxycytidine → dCMP → dUMP → deoxyuridine, the last reaction being the 5′-nucleotidase reaction. The enzymes that catalyze the first and the second reactions, dCK and dCMP deaminase, are elevated in cells infected by HSV. Because most of our assays utilize crude unfractionated extracts, the results that are presented earlier could alternatively be interpreted to be due to an increase in dCMP deaminase activity rather than the acquisition of dCD activity by the dCD$^-$ cells upon infection by herpesvirus. In order to distinguish between these possibilities, I used a mutant strain of HSV-1, TK21, that is unable to induce either dCK or TK activities in infected mouse cells (7). The cell line to be infected is the line 3T6-BCE, which is deficient in the dCK activity (14). Thus, when these cells are infected with TK21 virus, the first reaction in the alternative pathway described above is blocked. In this way, possibility that deamination might occur at the dCMP level is eliminated.

Table 2. Deoxycytidine deaminase (dCD) and deoxycytidine kinase (dCK) activity in 3T6-BCE cells infected with TK21 virus

Hour p.i.	Specific enzyme activity*	
	dCD	dCK
0	0.015	<0.001
3	0.016	<0.001
6	0.011	<0.001
12	0.015	<0.001
18	0.43	<0.001
24	2.33	<0.001
Control (3T6)	0.38	0.08

* Specific enzyme activity is expressed in nmol/min per mg of protein at 37°.

The experiment involved the infection of dCD$^-$ dCK$^-$ cell mutants with TK21 virus followed by assaying the infected cell extracts for both dCK and dCD activities. The results are summarized in Table 2. As expected, the TK21 mutant induced no dCK activity in the mutant cells, even after 24 hr of infection. By contrast, dCD activity in extracts of cells infected for 24 hr is present at very high levels, which are comparable to the levels induced by wild-type HSV. These results indicate that in order for the mutant cell to acquire dCD activity, simultaneous induction of dCK activity is not required. It can be concluded, therefore, that the induced deaminase activity as measured in the HSV-infected cells is indeed dCD activity, and not dCMP deaminase activity.

Thermostability of Virus-Induced Deoxycytidine Deaminase. It has previously been shown that virus-induced TK activity is more thermolabile than the TK activity of wild-type L cells (1). Similar experiments are carried out to determine whether virus-induced dCD activity has a different thermostability than that of host cell dCD activity. Cell extracts were prepared from LMTK$^-$ cells that were harvested 24 hr after infection with wild-type HSV-1. The extracts were partially purified on a Sephadex G-25 column to remove small effector molecules and the enzymes were then subjected to heat sensitivity tests at 60° and 56°. The results are shown in Fig. 3. At 60°, both cellular and virus-induced enzymes are inactivated to some extent, but the virus-induced enzyme is significantly more labile (*left*). At 56°, this difference in heat sensitivity becomes even more evident; the cellular enzyme remained completely active after 60 min of incubation, whereas 70% of enzyme activity in infected cells was inactivated (*right*).

In another experiment, the thermosensitivity of the dCD activity of 3T6-BCE cells infected with wild-type HSV was tested and similar results (not shown) were obtained. The results of heat inactivation experiments consistently indicate that the dCD activity in virus-infected mutant cells is markedly more thermolabile than the dCD activity in uninfected wild-type

(LMTK$^-$ or 3T6) cells, suggesting that the virus-induced dCD enzyme is very likely different from the enzyme that is present in uninfected mouse fibroblasts.

Actinomycin D Inhibition of dCD Induction. Actinomycin D experiments were performed to determine whether viral induction of dCD requires *de novo* DNA-dependent RNA synthesis. The drug is added to HSV-infected 3T6-BCE culture, at 30 min, 5 hr, or 10 hr p.i. to a final concentration of 0.5 μg/ml. At this concentration, 90% of total RNA synthesis is inhibited as measured by [^{3}H]uridine incorporation into RNA by the cells. At intervals, cells were harvested and the dCD activities were assayed. The results, presented in Fig. 2, show that the dCD induction by viral infection is completely inhibited when actinomycin D is added at 30 min p.i., suggesting that *de novo* RNA synthesis is necessary for the appearance of virus-induced dCD activity. When the drug is added later in the infection cycle (5 or 10 hr p.i.), further increase in enzyme activity is blocked such that the activity remains at a plateau level.

The results of actinomycin D experiments together with those of thermosensitivity tests suggest either that the viral genome specifies the structure of the induced dCD molecules or that HSV, upon lytic infection, induces the expression of a previously repressed cellular gene.

Electrophoretic Separation of Human Cellular and Viral Enzyme. Attempts to separate the virus-induced dCD from mouse dCD by Cellogel electrophoresis at three different pH values, 7.6, 8.6, and 6.8, proved unsuccessful (Fig. 4, *A* and *B*). Because we found previously that mouse dCD and human dCD were readily separated by Cellogel electrophoresis (14), we tested extracts from the human line HEp-2, infected with HSV-1, by electrophoresis on Cellogel. This human line characteristically contains high levels of dCD activity (18). Nonetheless, in the HEp-2 line we observed a 2- to 3-fold increase in dCD specific activity after HSV infection. The results of Cellogel electrophoresis (Fig. 4, *C* and *D*) indicate that the virus-infected cell extracts produce a new dCD peak that comigrates with the mouse dCD marker, and also with the enzyme in the extracts of mouse cells infected with HSV-1. Therefore, it is very likely that the new peak that appears in both the mouse mutant line infected by HSV-1 and the human line infected by HSV-1 is a protein encoded by the viral genome.

DISCUSSION

The results presented in this paper clearly show that HSV-1 induces dCD enzyme activity upon lytic infection of a mouse cell line deficient in dCD. Activity of this enzyme is also elevated, although to a lesser extent, in wild-type cell lines of various species, including a human and a Syrian hamster cell line, when they are infected by HSV-1.

An important question is whether the induced enzyme is coded by the viral or the cellular genome. Evidence supporting the contention that the induced dCD is of viral origin comes from several sources. First, it is shown that the induction of dCD in the dCD$^-$ mutant cell line is inhibitable by actinomycin D, which indicates that the induced enzyme is probably not a result of the modification of previously synthesized cellular enzyme molecules. The possibility that the induced enzyme is derived from the reactivation of the mutationally inactivated cellular dCD gene is rendered unlikely by the observation that the heat sensitivities of the induced and cellular enzymes are different. Last, there is a remote possibility that lytic infection by HSV can activate the expression of a different cellular dCD gene that is normally not expressed in cultured fibroblasts. However,

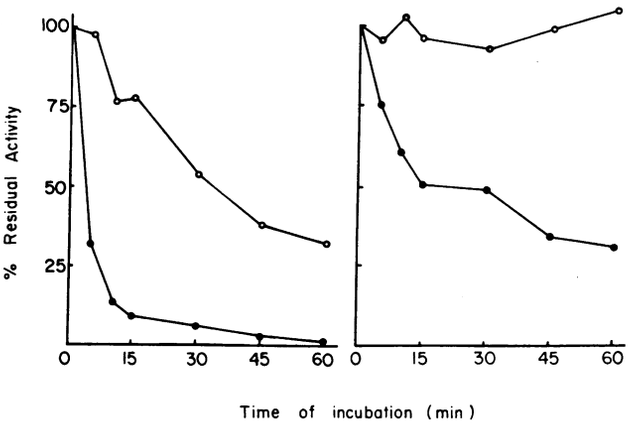

FIG. 3. Thermosensitivity test of cellular (LMTK$^-$) and virus-induced dCD. The enzyme activity that remains after heating is expressed as percent of the original, unheated activity. *Left*, 60°; *right*, 56°. Enzyme in uninfected cells, (○); enzyme in infected cells, (●).

because the induced dCD enzyme always has a characteristic electrophoretic mobility regardless of the species of host cells that is infected, it is highly unlikely that this possibility is true. Taken together, our data strongly suggest, though do not prove, that the HSV-induced dCD is coded by the viral genome.

The enzyme dCD, if it is indeed coded by the viral genome, could be a useful selective marker for selecting dCD$^+$ cells from deficient cells after infection by UV-inactivated HSV-1 virus. The basis for the selection method, described in detail elsewhere (14), is that viral dCD, like the cellular enzyme, converts 5-methyldeoxycytidine into thymidine (Chan, unpublished). Thus, in the presence of aminopterin, which blocks *de novo* thymidylate synthesis, dCD$^+$ cells can utilize 5-methyldeoxycytidine as the exogenous source of thymidylate, whereas dCD$^-$ cells are unable to do so. The selective marker that has already been successfully used for herpes viral gene transfer experi-

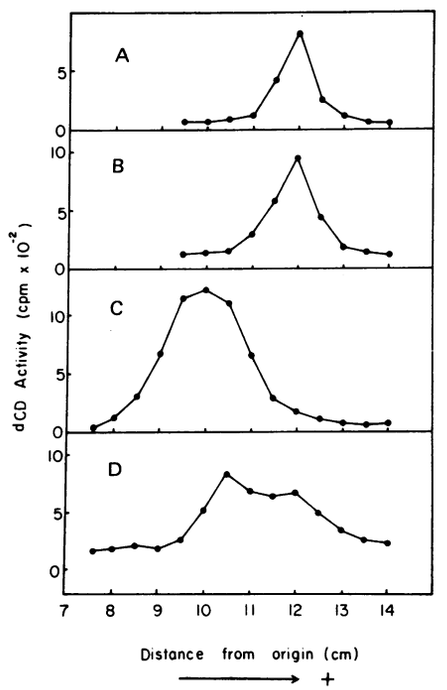

FIG. 4. Cellogel electrophoresis of deoxycytidine deaminase. (*A*) Mouse cell extract, 3T6; (*B*) Extract from 3T6-BCE cells infected with HSV-1; (*C*) human cell extract, HEp-2; (*D*) extract from HEp-2 cells infected with HSV-1.

ments is thymidine kinase (1, 9). Having dCD as an additional selective marker would permit us to investigate further this unique viral gene transfer system.

The function of the induced dCD with regard to viral growth is not well understood. In the case of cellular dCD, it has been proposed, on the basis of allosteric inhibition of its activity by dTTP, that the enzyme probably has a role in the synthesis of thymidylate *via* the salvage of deoxycytidine (25). The virus-induced dCD may have a similar role. This view is supported by the parallel increases in TK and dCD activities in HSV-infected LMTK$^-$ cells (Table 1). It has been reported that all deoxynucleoside triphosphates pools increase in cells after infection with HSV and the increase in the dTTP pool is much higher than that in the dCTP pool (26–28). High levels of dCD, which converts deoxycytidine to thymidine monophosphate, might account for this disproportionately high level of dTTP in the infected cells.

In view of the induction of dCD by HSV-1, the kinetic data with regard to virus-induced TK should be more carefully evaluated. It has been reported that the viral TK phosphorylates thymidine and deoxyuridine as well as deoxycytidine, and hence is called "deoxypyrimidine kinase," whereas the host cell cytosol enzyme phosphorylates only the first two substrates (29). However, most of the experiments designed for characterizing the viral deoxypyrimidine kinase used only partially fractionated extracts prepared from virus-infected cells. In such an extract, there is the possibility that deoxycytidine can be deaminated by dCD to form deoxyuridine and deoxyuridine can be further phosphorylated to dUMP by the action of TK. Thus, the usual assay method for dCK, where the binding of nucleoside monophosphates to DEAE-paper discs is utilized, is no longer a reliable measurement for dCK. Therefore, to more accurately study the substrate specificity and kinetic parameters of the virus-induced deoxypyrimidine kinase, proper precautions should be taken to ensure that the enzyme preparation is devoid of contaminating dCD (30).

The finding reported here raises a question concerning the effectiveness of current methods used for the treatment of herpes infection. It is known that host dCD deaminates the deoxycytidine analogue cytosine arabinonucleoside to its inactive form, uracil arabinonucleoside (31). If the viral enzyme also deaminates cytosine arabinonucleoside, which is commonly used in chemotherapy of herpes infection, then the efficiency of the drug against herpes infection would be substantially reduced. Therefore, it is possible that the additional presence of an inhibitor of dCD [such as tetrahydrouridine (32)] would greatly enhance the therapeutic value of cytosine arabinonucleoside for the treatment of herpes infection.

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