Thymidine Kinase from Normal, Simian Virus 40-Transformed and Simian Virus 40-Lytically Infected Cells

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Simian virus 40 (SV40) infection of human diploid cells failed to cause an enhanced production of thymidine kinase during the first 10 days after infection. Thymidine kinase activities from extracts of SV40-transformed cultures (human or simian) were considerably higher than the activity levels in extracts from the normal cells of origin. In addition, whereas the kinase activities obtained for human diploid cultures decreased as the cell sheet became confluent, the kinase activities for SV40-transformed human cells remained high after confluence was reached. Antisera obtained from hamsters bearing SV40 or adeno-7-SV40 hybrid virus tumors selectively inhibited enzyme from transformed sources (human or simian). Also, the antisera selectively inhibited enzyme extracted from SV40-lytically infected monkey cells. Sera from normal animals or from hamsters bearing polyoma tumors failed to inhibit enzymes from normal, SV40-transformed, or SV40-lytically infected cells. The Michaelis constant of partially purified enzyme from SV40-transformed cells was two to five times as high as that obtained for partially purified enzyme from human diploid cell cultures.

Simian virus 40 (SV40) enhances thymidine (TdR) kinase activity in abortively infected mouse cells (19) and in cells which subsequently undergo lysis, such as African green monkey kidney (GMK) cells (17) and a stable line of GMK, CV-1 (13, 18). The enzyme produced after infection of CV-1 cells has some characteristics different from those of TdR kinase obtained from uninfected cells (18). Human diploid cells infected with SV40 show comparatively little virus replication or cytopathic changes, and eventually some of the cells transform (4). The transformed cells become predominant in the culture, and in comparison to normal diploid cells, are altered in their growth, morphological, and chromosomal characteristics (21, 26, 30). It was of interest to determine whether SV40 enhanced TdR kinase activity in the early stages after infection in a transformable system. In addition, the specific activities and characteristics of TdR kinases obtained from normal and from transformed cells were compared.

In studying the characteristics of the enzymes, special attention was paid to possible antigenic differences. Immunologically detectable differences between the TdR kinases obtained from normal cells and those obtained from virus-

infected cells have been demonstrated in several lytic cell-virus interactions, such as L cells infected with vaccinia, rabbit kidney cells infected with pseudorabies virus, and several cell lines infected with herpesvirus (11, 15, 20). Also, herpesvirus infection causes the production of a specific deoxyribonucleic acid (DNA) polymerase which is immunologically distinct from normal cell polymerase (14, 24). SV40 is capable of inducing an immunologically distinct protein as evidenced by the SV40 T antigen (referred to here as induced complement-fixing antigen, ICFA) which is found in cells in a lytic cell-virus interaction and in all SV40-transformed cells (2, 3, 9).

MATERIALS AND METHODS

Cells. The human diploid cell strain WI-38 was used from the 18th to the 48th passage. An SV40-transformed line, W18VA-2, was used (26). This line was virus-free and contained SV40 T antigen in 100% of the cells. The transformed line is subsequently referred to as VA-2.

GMK cells and two lines isolated from GMK were also used. One of these lines, CV-1, was an isolate from normal, uninfected GMK cells (13) and is susceptible to lysis by SV40 (5, 18). The GT-LP-4 line of cells is an SV40-transformed line of GMK. Cultures from the GT-LP-4 line were virus-free and

100% of the cells contained the SV40 T antigen. The techniques used for developing this line included the infection of replicating GMK cells with SV40 and repeated subculturing of the cells prior to their reaching confluence. The details of this procedure have been described (4).

All cells were cultured in stoppered 32-oz (0.9-liter) bottles or in plastic petri dishes maintained in an atmosphere of 4% CO₂ in air. The growth medium for all types of cells was Earle's salt solution containing the mixture of amino acids and vitamins recommended by Eagle (6), and 10% serum (EE₁₀). Streptomycin (100 µg/ml), penicillin (100 units/ml), and kanamycin (166 µg/ml) were used in the medium.

Virus. A large-plaque variant of the RH911 strain, termed LP-4, was used. The virus was passaged five times in CV-1 cells. The pool used in these experiments contained 10^{6.5} plaque-forming units (PFU) of virus per milliliter and is referred to as LP-4, CV pool 2.

Enzyme studies. From 10 × 10⁶ to 60 × 10⁶ cells were used for each assay. The cells were removed from glass or plastic with 0.25% trypsin in 0.1% ethylenediaminetetraacetate. The trypsin action was stopped by dilution with an equal volume of EE₁₀ medium. All subsequent steps were done at 4 C. The suspended cells were centrifuged, washed once with cold phosphate-buffered saline, and then suspended in six volumes of sonic-treatment buffer which consisted of 0.15 M KCl, 0.003 M 2-mercaptoethanol, and 0.01 M tris(hydroxymethyl)aminomethane at pH 8.0. The suspension was subjected to ultrasonic treatment for 45 to 85 sec (Raytheon sonic oscillator model DF 101, 10 kc). Cell disintegration was monitored by microscopic examination. Supernatant fractions (enzyme extracts) were obtained by centrifugation of the samples at 32,000 × g for 1 hr. TdR kinase activity was determined as described by Kit et al. (15, 16, 18) by using tritiated deoxyuridine as substrate, and by measuring the amount of deoxyuridine-5'-monophosphate (dUMP) formed. Protein content was determined by the method of Lowry et al. (23).

Sera. One pooled serum (A) obtained from hamsters bearing virus-free SV40-induced tumors, was kindly supplied by K. K. Takemoto (National Institutes of Health). The serum was obtained approximately 6 months after inoculation of SV40-induced hamster tumor cells and several months after the excision of the initially developing tumors. Serum B, obtained from hamsters bearing tumors induced by adenovirus 7-SV40 hybrid virus (12) was supplied by D. Porter (Wistar Institute); and serum C, from one hamster with a primary SV40 tumor, was supplied by B. DeVillano (Wistar Institute). Several other pooled and individual sera obtained from hamsters with SV40 or polyoma tumors were obtained from R. Gilden (Flow Laboratories), A. J. Girardi (Wistar Institute), K. K. Takemoto, D. Porter, and B. DeVillano.

Enzyme-antiserum reaction. Equal volumes of enzyme extract and serum were mixed and placed at either 4 or 38 C. The samples at 38 C were mixed continuously during incubation and were then

placed at 4 C for 16 to 24 hr. In some early experiments the enzyme extract-serum mixtures were centrifuged at 32,000 × g (4 C) prior to assay for TdR kinase activity.

Enzyme purification. The techniques used for TdR kinase purification were similar to those employed by Kit et al. (18). The enzyme extracted after ultrasonic treatment was centrifuged at 36,000 rev/min (model L Spinco centrifuge with 40 rotor) for 90 min. The enzyme was precipitated from the supernatant fraction (S-1) by the addition of 0.23 g of ammonium sulfate per ml of solution. After stirring the suspension for 30 min, the material was centrifuged and the precipitate was redissolved in sonic-treatment buffer at a concentration of 1.0 to 1.6 mg of protein per ml. Calcium phosphate gel was mixed with the enzyme solution at a ratio of 2 mg (dry wt) per mg of protein for 8 min at 4 C. The mixture was centrifuged at 12,000 rev/min for 10 min; the supernatant fraction contained approximately 10% of the protein and approximately 50% of the enzyme activity obtained in S-1. Thus, there was an increase in specific activity of from four- to sevenfold.

Mitotic index. Cultures maintained on cover slips were examined by phase-contrast microscopy to determine the proportion of cells in mitosis.

autoradiography. Sixty min after the addition of 2.5 µc/ml of ³H-thymidine (6 c/mmole) per petri dish (4 ml of medium), monolayer cultures maintained on cover slips were washed, fixed, and treated as described previously (4).

Immunofluorescence and complement-fixation tests. Immunofluorescence and complement-fixation (CF) tests were performed as described previously (4, 9).

RESULTS

Effect of SV40 infection on the TdR kinase activity in WI-38 cultures. In a series of kinetic studies, cultures of WI-38 cells from the 18th to 48th passage were infected with SV40 LP-4, CV pool 2 at virus-to-cell ratios of between 4.8:1 and 480:1. Cultures used for control points were mock-infected with spent medium and then treated in the same manner as virus-infected cultures. At various times during the first 10 days after infection, cultures were harvested and TdR kinase was extracted and analyzed by techniques described in Materials and Methods. In all studies, the kinetic curves obtained for enzyme activities of infected cells were similar to those obtained for control cultures. Thus, enzyme was not induced in SV40-infected diploid cultures.

In several experiments, sister cultures infected at the same multiplicities as those used for enzyme extractions were examined for the development of ICFA by fluorescent antibody techniques. In all instances, less than 5% of the cells were positive for ICFA. CV-1 cells infected under similar conditions, and examined from 24 hr postinfection until extensive cytopathic effect occurs, yield TdR kinase activities in extracts

FIG. 1. Cultures of WI-38, passage 20, and VA-2 were trypsinized and subcultured on day 0 into 32-oz bottles for thymidine kinase activity analysis, or into petri dishes with cover slips for analysis of mitotic indices and for ^3H -thymidine incorporation studies. Cells were placed in the different culture vessels at the same cell density per surface area. Analyses were made at daily intervals. The techniques for harvesting and analysis for enzyme activity are described in Materials and Methods. Cover slips were removed at indicated times and examined under phase contrast to determine mitotic index. Tritiated thymidine (6 c/mmole) was added at the rate of 10 μc per petri dish prior to harvesting cover slips used for autoradiography studies. The cover slips were harvested and processed as described. Kinase, solid line; labeled cells, broken line; mitotic index, diagonally broken line.

from infected cultures from 2 to 20 times those obtained from control cultures (5, 17, 18). In addition, under these conditions 100% of CV-1 cells are positive for ICFA by 48 hr postinfection (Carp, unpublished data).

TdR kinase activities in VA-2 and WI-38 cultures. It was of interest to compare kinase activity levels in transformed and normal cells. TdR kinase activities were determined in WI-38 and VA-2 cultures at daily intervals after subcultivation. In addition, at each time, sister cultures were analyzed for mitotic index and by autoradiography for the proportion of cells incorporating ^3H -thymidine after a 1-hr labeling period. The results are shown in Fig. 1. In diploid cultures the level of TdR kinase activity peaked on the 2nd day, and then, as the cells began to fill the surface areas of the culture vessels and their replication frequency began to decrease because of contact inhibition, the level of kinase activity decreased. The cells formed a confluent monolayer by the 4th day after subcultivation. Contact inhibition of replication was reflected in the depression in mitotic index and in the proportion of cells incorporating ^3H -thymidine. This pattern

of TdR kinase activity is very similar to that obtained for LM and L cells (16, 31). In contrast, the amount of TdR kinase in VA-2 cells did not show a similar pattern in relation to subcultivation, and to the occurrence of confluent cultures (day 4); the activities were considerably higher than the activities obtained in diploid cultures. In 30 separate extractions, TdR kinase activities were always higher in VA-2 than in diploid cell extracts. The mean activity values and standard deviations of the means for VA-2 and WI-38 cultures were 5.12 ± 2.73 and 0.78 ± 0.58 , respectively (activity units = μmoles of dUMP formed per μg of protein in 10 min at 38 C). TdR kinase activities from another SV40-transformed human line WI-38VA-13 (10), and from a transformed GMK line GT-LP-4, were higher than those obtained for the normal parental cultures. Similar results were obtained for SV40-transformed and normal mouse cultures by Kit et al. (19).

Effect of several antisera from hamsters bearing SV40 tumors on TdR kinase from VA-2 and WI-38 cultures. Enzyme extracts from WI-38 and VA-2 were mixed with an equal volume of

serum from SV-40 or polyoma tumor-bearing animals or with serum from normal hamsters. The mixtures were shaken for 45 min at 38 C, placed at 4 C overnight, and then analyzed for enzyme activity. Enzyme extracts mixed with sonic-treatment buffer (or with normal serum known to be noninhibitory from previous tests done in parallel with sonic-treatment buffer) were similarly treated and used as controls. Of 15 sera from SV40 tumor-bearing animals, 10 had a pronounced inhibitory effect on enzyme from VA-2 cells. Several of these sera also inhibited activities of enzyme from WI-38 (see serum B below), but to a much smaller extent than the inhibition of enzyme activity from VA-2. In contrast, 19 sera from normal hamsters and 4 sera from hamsters bearing polyoma tumors did not inhibit enzyme from either source. The inhibition of VA-2 enzyme activity by serum from hamsters bearing SV40 tumors was most pronounced with two pooled sera (A and B) and an individual hamster serum termed C. The anti-ICFA titers in CF tests of sera A, B, and C were ≥ 512 , ≥ 160 , and ≥ 8 , respectively. The effects of these three sera were studied further, and results are described in subsequent sections.

Results obtained with antiserum A are shown in Table 1. This antiserum inhibited the activity of enzyme extracted from the SV40-transformed cells (VA-2), but had no effect on the activity of enzyme from WI-38 cultures. Dilution of serum led to a reduced neutralizing effect, and in addition, the antiserum was more effective in neutralizing enzyme activity after dilution of the enzyme.

In each of several mixtures of antiserum A and enzyme extract, a pellet was found after centrifugation. No enzyme activity was found in the pellets. The pellets persist after treatment of the serum-enzyme mixtures with an equal volume of more antiserum A. The second treatment with serum leads to a further reduction in enzyme activity. Clarification of serum prior to use by centrifugation at $32,000 \times g$ for 1 hr, which in large part eliminated pellet formation, did not affect the potency of the antiserum.

It was of interest to determine whether the reduction in enzyme activity in VA-2 extracts was related to nonspecific adsorption to, or trapping in, antigen-antibody complex. For this purpose, TdR kinase from VA-2 cultures was mixed equally with either normal hamster serum or with a 20% suspension of a precipitate formed by mink-immune γ -globulin and rabbit serum containing anti-mink γ -globulin antibodies (obtained from D. Porter, Wistar Institute). The mixtures were treated in the same manner as the above serum-enzyme mixtures. There was no

TABLE 1. *Effect of antiserum A on thymidine kinase extracted from WI-38 and VA-2 cultures*

Expt	Enzyme ^a		Sera ^a		
	Origin	Dilution	Control	Immune (A) ^b	
				UN	1-4
1	WI-38	UN	0.34 ^c	0.56	—
	VA-2	UN	4.72	3.12	5.22
	VA-2	1:4	7.02	2.48	4.90
2	WI-38	UN	0.56	0.56	—
	VA-2	UN	6.58	2.82	—
3	WI-38	UN	0.46	0.49	—
	VA-2	UN	2.81	2.07	—
	VA-2	1:8	3.47	1.3	—
4	WI-38	UN	1.16	1.22	—
	VA-2	UN	7.62	4.68	—
5	WI-38	UN	0.50	0.69	—
	VA-2	1:6	3.40	2.19	—

^a Equal volumes of enzyme extract and serum were mixed at 38 C for 45 min. The samples were placed at 4 C for 18 hr and then were assayed for residual enzyme activity, or, in some experiments, centrifuged at $32,000 \times g$ prior to assay. UN = undiluted.

^b Immune serum A was obtained from hamsters bearing virus-free SV40 tumors.

^c Units refer to μ moles of dUMP formed per μ g of protein in 10 min at 38 C.

difference in enzyme activity in samples obtained from enzymes mixed with antigen-antibody complex and with normal hamster serum.

In neutralization experiments with antiserum B (Table 2), TdR kinase activity from both sources was affected, but the reduction in kinase activity from VA-2 extracts was much more pronounced than that from WI-38 extracts. Furthermore, upon dilution of the antiserum, the effect upon enzyme from diploid cells could be eliminated, whereas the neutralizing effect on enzyme from VA-2 was retained, although to a reduced degree. Experiments 4 and 5 of Table 2 show results obtained by using partially purified TdR kinase. The enzyme extracts, purified by high-speed centrifugation, precipitation with $(\text{NH}_4)_2\text{SO}_4$, and negative adsorption to CaPO_4 gel (see Materials and Methods and reference 18), were mixed with antiserum and tested as in the above sections with the following exception. By using very dilute enzyme or partially purified material, we eliminated centrifugation at $32,000 \times g$, because we found that high-speed centrifugation of enzyme in a dilute or partially

TABLE 2. *Effect of antiserum B on thymidine extracted from WI-38 and VA-2 cultures*

Expt	Enzyme ^a		Sera ^a					
	Origin	Dilution	Control	Immune (B) ^b				
				UN	1-3	1-6	1-12	1-24
1	WI-38	UN	1.16 ^c	0.60	—	—	—	—
	VA-2	UN	7.62	0.38	—	—	—	—
2	WI-38	UN	0.50	0.22	—	—	—	—
	VA-2	1:6	3.40	0.28	—	—	—	—
3	WI-38	UN	0.67	—	0.43	—	—	—
	VA-2	1:6	2.43	—	0.00	—	—	—
4 ^d	WI-38	UN	1.31	—	—	1.32	—	—
	VA-2	1:5	8.2	—	—	2.86	6.46	8.2
5 ^d	WI-38	UN	1.02	—	0.75	—	—	—
	VA-2	1:5	12.066	—	0.0	—	—	—

^a Enzyme and serum mixed as described in Table 1. UN = undiluted.

^b Immune serum B was obtained from hamsters bearing adeno 7-SV40 hybrid tumors.

^c Units refer to $\mu\mu$ moles of dUMP formed per μ g of protein in 10 min at 38 C.

^d Enzyme extracts purified four- to sevenfold prior to mixing with antiserum.

purified state led to pronounced nonspecific inactivation. The results (Table 2) show that partially purified TdR kinase prepared from VA-2 cells was inactivated by antiserum B, whereas enzyme from WI-38, purified to the same extent, was comparatively unaffected.

The Michaelis constants (K_m) of the partially purified TdR kinases were determined in three separate experiments by the method of Lineweaver and Burk (21), in which values for the concentration of dUR divided by the velocity were plotted against the dUR concentration. Four concentrations of dUR ranging from 12.5 to 100 μ M were used to determine the K_m of enzyme from diploid cells, whereas five concentrations ranging from 12.5 to 200 μ M were used for enzyme from VA-2 cultures. The mean K_m value and standard deviation of the mean for enzyme from VA-2 cells was $5.64 \pm 0.65 \times 10^{-5}$ M and for enzyme from WI-38 cells was $1.47 \pm 0.14 \times 10^{-5}$ M. The mean V_{max} and standard deviation of the mean for enzyme from WI-38 was 1.66 ± 0.31 and for enzyme from VA-2 it was 52.77 ± 13.7 (units are $\mu\mu$ moles of dUMP/ μ g of protein in 10 min at 38 C).

Antiserum B (1 ml) was mixed with an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate

TABLE 3. *Effect of antiserum A on thymidine kinase extracted from GMK and GT-LP-4 cultures*

Expt	Origin of enzyme	Sera ^a	
		Control	A
1	GMK	0.74 ^b	0.74
	GT-LP-4	3.06	1.26
2	GMK	0.34	0.38
	GT-LP-4	2.0	1.38
3	GMK	0.32	0.34
	GT-LP-4	1.49	1.03

^a Enzyme and serum mixed as described in Table 1.

^b Units refer to $\mu\mu$ moles of dUMP formed per μ g of protein in 10 min at 38 C.

that formed was collected by centrifugation, washed several times with 50% saturated ammonium sulfate, dissolved in 1 ml of sonic-treatment buffer, and dialyzed for 3 days against four changes of sonic-treatment buffer. The dialyzed portion was as effective as untreated serum in neutralizing enzyme from VA-2 cells.

Effects of antisera A on enzyme extracts from GMK and GT-LP-4 cells. The GT-LP-4 line was obtained after SV40 infection of GMK cells. The transformed line, which was virus-free and positive for ICFA, was obtained as described in Materials and Methods. Serum A was used in neutralization tests with TdR kinase extracted from the two types of cells (Table 3). It was found that the antiserum from tumor-bearing hamsters neutralized enzyme extracted from the transformed simian cells (GT-LP-4), whereas the sera had little or no effect on enzyme from normal cells (GMK).

Effect of antisera A, B, and C on the enzyme extracted from mock-infected and SV40-infected CV-1 cells. CV-1 cells were infected with SV40 LP-4, CV pool 2, at a multiplicity of between 50:1 and 80:1. Infected and control (mock-infected with spent medium) cultures were harvested 48 hr postinfection. The TdR kinases were then used in neutralization tests with normal hamster serum or with serum A, B, or C. The results, shown in Table 4, indicate that the sera from tumor-bearing animals reduced the TdR kinase activity of infected CV-1 extracts to a greater extent than enzyme activity from control CV-1 cells. Antiserum C was also found to be effective in neutralizing enzyme activity in ex-

TABLE 4. *Effect of antisera A, B, and C on thymidine kinase extracted from SV40-infected and control CV-1 cultures*

Expt	Serum ^a	Dilution ^b	Enzyme ^a extracted from	
			Control CV-1	SV40-infected CV-1
1	Control	UN	0.68 ^c	2.20
	A	UN	0.70	1.69
	B	1:3	0.76	1.06
2	Control	UN	0.29	2.43
	B	1:6	0.54	1.29
3	Control	UN	0.70	3.30
	C	UN	1.22	0.90

^a Enzyme and serum mixed as described in Table 1. Sera A and C obtained from hamsters bearing virus-free SV40 tumors. Serum B from hamsters bearing adeno 7-SV40 hybrid tumors.

^b UN = undiluted.

^c Units refer to μ moles of dUMP formed per μ g of protein in 10 min at 38 C.

tracts from VA-2 cells, whereas it was without effect on enzyme from WI-38 cells.

DISCUSSION

Failure to demonstrate an enhancement of TdR kinase activity in SV40-infected WI-38 cultures was probably related to the small proportion of cells that were productively infected. If the induction of ICFA is taken as a measure of the proportion of productively infected early-passage human diploid cells, then a very small percentage of cells are engaged in the synthesis of virus products (see Results and reference 4). If a similar proportion of cells were induced to produce kinase, the increase in activity would be so slight as to be undetected by the assay methods used.

There are a number of possible explanations for the comparatively high levels of TdR kinase activity in transformed cultures. It has been shown that kinase activity is correlated positively with the rate of cell replication (16, 31). There is reduced contact inhibition of replication in transformed cells; they therefore continue to replicate after a confluent monolayer has formed. Higher kinase activities in transformed cultures might reflect this continued replication. However, at the points of maximal diploid cell replication (2 to 3 days), there is a greater difference in kinase activities for the two cell types than there is for the values of either mitotic indices or thymidine-incorporating cells. Another possible explana-

tion for the high kinase activities in VA-2 cells is that the metabolic control mechanisms of transformed cells may have been upset during or following transformation by SV40. Finally, there is the possibility that there are two distinct enzymes in SV40-transformed cultures, one induced by the virus genome and the other of cellular origin, and that these are produced concurrently. This last hypothesis is supported by the fact that the TdR kinase activities found in transformed cells and in SV40-infected CV-1 cells were partially neutralized by antisera to primary SV40 tumors.

Cells transformed by SV40, polyoma, or adenovirus have been found to contain considerable amounts of messenger RNA which hybridizes with the DNA of the transforming virus (1, 7, 27). Thus, transformed cells probably have the potential to produce a number of virus-specific proteins. The specific neutralization of TdR kinase obtained from several transformed cell lines by antisera from hamsters bearing SV40-induced tumors indicates that an immunologically distinct enzyme may be produced in transformed cells. The fact that these antisera also neutralize TdR kinase from SV40-infected CV-1 cells suggests that the enzyme that is neutralizable in transformed cells and in lytically infected cells is dependent on virus genome for its development. The persistence of an SV40-specific protein in transformed cells has been documented in the case of the ICFA (9).

The immunological distinctiveness noted in TdR kinase from SV40-transformed and infected cells suggests that the virus codes for the enzyme. There are, however, several alternative explanations. The antigenic difference may have arisen from a physical or chemical alteration in the enzyme from normal cells. Since enzymes from transformed cells were immunologically distinct, the cause of the alteration, or the alteration itself, would have to be reproduced in conjunction with cell replication. Another possible explanation of the immunological difference is that SV40 infection derepresses the synthesis of an isozyme of TdR kinase coded for, but not normally synthesized in, uninfected cells (25). This derepressed state would have to exist both in SV40-transformed cells and in lytically infected cells.

The development, in hamsters with tumors induced by adenovirus 7-SV40 hybrid virus (12), of antisera (serum B) capable of neutralizing TdR kinase obtained from SV40-transformed or lytically infected cells, indicates that the information concerned with the production of SV40-specific TdR kinase is present in tumors of "hybrid" virus origin. It has been demonstrated that

"hybrid" virus can induce TdR kinase in a lytic cell-virus interaction (17).

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