Marker Rescue with Ultraviolet-Inactivated Influenza Virus

A. SUGIURA¹ AND M. UEDA

The Institute of Public Health, Minato-ku, Tokyo 108, Japan

Received for publication 27 January 1971

Influenza A1/CAM virus undergoes abortive growth in FL cells, whereas X-3311, a recombinant virus clone from the cross between CAM and NWS, is capable of complete replication in FL cells and plaque formation on FL monolayers (FL-marker). Clone 1-5C-4 cells are permissive for both viruses. When either clone 1-5C-4 or FL cells, which were mixedly infected with both ultraviolet-irradiated X-3311 and active CAM viruses, were plated on FL monolayers, infective centers far exceeding in number those expected from the surviving X-3311 virus were observed, i.e., the FL-marker of the irradiated parent was rescued. The significantly lower radiation sensitivity of FL-marker than that of infectivity indicates that only part of the genome is responsible for the FL-marker. The capability of X-3311 virus to produce NP antigen and its infectivity were lost at the same time when the former was assayed under the condition of low multiplicity of infection. This suggests that only infectious virus is capable of producing NP antigen and that no stepwise inactivation of the viral genome occurs. It is also suggested that any lethal ultraviolet damage inflicted upon the viral genome prevents the expression of the portion of the genome coding for NP antigen.

Genetic functions may be retained by influenza virus rendered noninfectious by ultraviolet (UV) irradiation. Those genes which have survived radiation damage are reactivated efficiently under the condition of high multiplicity of infection (1) or after co- or superinfection with heterologous influenza virus (3, 5, 8). We have tried to dissociate the inactivation of individual genes, which are expected to have smaller target size than the whole genome, from that of infectivity which presumably requires the whole intact genome. For this purpose, two markers that can be studied easily were chosen. One property necessary was the capacity to synthesize the NP antigen, which can be detected by immunofluorescence. The second was the capacity to produce mature infectious progeny in human amnion FL cells. Infection of FL cells with most influenza virus strains, including A1/CAM, results in abortive replication in which normal amounts of NP antigen and hemagglutinin are produced, yet very little infectious virus is made (6). A0/NWS and X-3311 virus, a recombinant virus clone obtained from the cross of NWS and CAM, complete their entire growth cycle in FL cells and produce

plaques on monolayers of FL cells. The property of a virus to produce mature infectious progeny in FL cells is referred to as the "FL-marker." The FL-marker has been shown to be readily transferable from one virus to the other during the act of recombination (K. Tobita, Arch. Gesamte Virusforsch, *in press*). It was expected that, if the putative gene(s) governing the FL-marker has survived UV radiation, the marker should be rescued by the superinfection with an infective heterologous virus lacking the FL-marker. In the present study, infection was carried out with suspended cells and the occurrence of infection was determined by demonstrating either plaque-forming infective centers or NP antigen-containing cells. Infection of suspended cells gave more reproducible results than that of monolayer cells, probably because viral adsorption to cells occurs with higher efficiency and greater uniformity in the former case.

MATERIALS AND METHODS

Cell cultures. The human conjunctival cell line clone 1-5C-4 (4) and the human amnion cell line FL were used. Media employed for propagation and maintenance of cells have been described previously (6).

Virus strains. The CAM strain of influenza A1 virus and the X-3311 virus were used. X-3311 is a virus clone obtained from the cross between A0/NWS and A1/

¹ Present address: Department of Microbiology, Mount Sinai School of Medicine of the City University of New York, New York, N.Y. 10029

CAM strains. This virus possesses the hemagglutinin of NWS serotype and is capable of replicating in FL cells (FL-marker) and plaquing on both clone 1-5C-4 and FL cells with equal efficiency, whereas CAM virus plaques on clone 1-5C-4 cells but not on FL cells (Fig. 1). Viruses were grown in chick embryos at limiting dilution. All virus stocks were stored at -80°C .

Antisera. Hyperimmune rabbit anti-NWS serum and guinea pig serum specific for the NP antigen of influenza A virus were prepared by methods previously

described (6). Fluorescein isothiocyanate was conjugated to the latter serum by a method described in an earlier report (7).

Titration of infectious virus. Infectivity was determined by titration of plaque-forming infective centers as follows. A suspension of 10^6 freshly trypsinized cells was mixed with 0.2 ml of appropriately diluted virus. Virus was diluted so that the multiplicity of infection (MOI) was less than 0.05 plaque-forming unit (PFU)/cell. Virus adsorption to cells was allowed to proceed for 30 min at 37°C with occasional shak-

FIG. 1. Plaques of CAM and X-3311 viruses. (A) CAM, (B) X-3311 plaques after 5 days of incubation. Left, clone 1-5C-4; right, FL monolayer cultures.

ing. More than 95% of the input virus was adsorbed during this period. Cells were washed and resuspended in 1 ml of fresh maintenance medium. Serial dilutions were prepared in fivefold steps, and 0.1 ml of the cell suspension of the appropriate dilution was deposited on drained monolayer cultures in a 6-cm petri dish. Inoculated cells were spread over the entire surface by shaking of the dish. After incubation at 36°C for 30 min for attachment of cells to the monolayer, agar overlay medium was gently added. The addition of secondary overlay and counting of plaques were done in the same way as in the conventional plaque assay (6). The efficiency of plating, i.e., the ratio of plaque number to the number of infected cells plated, was 100%. Infectivity thus measured was consistently 1.5- to 2-fold higher than that determined by the conventional plaque assay. Clone 1-5C-4 cells were used for titration of CAM virus, and FL cells were used for titration of X-3311 virus.

Rescue of FL-marker. A slight modification of titration of infective centers was used. Suspended cells were infected first with X-3311 virus, either active or irradiated for various intervals, at a MOI ranging from 0.01 to 0.1 PFU/cell in terms of preirradiation titer. After adsorption, cells were pelleted by low-speed centrifugation, and they were then exposed to either 0.2 ml of CAM virus or maintenance medium. At the end of a 30-min adsorption period, cells were again spun down and resuspended in 1 ml of fresh maintenance medium. Cells were plated onto FL monolayer cultures. Subsequent procedures were the same as described in the infectivity titration.

Counting of NP antigen-containing cells. Infection of suspended FL cells with X-3311 virus was performed by the same procedure as employed for virus titration, except that the virus adsorption period was extended to 60 min at 37°C for the purpose of minimizing the neutralization of adsorbed, but still unclipped, virus particles by the subsequent antiserum treatment. Cells were centrifuged and were resuspended in growth medium containing anti-NWS serum at a final concentration of 1:50, so that each 0.3 ml contained 4×10^4 cells. A 0.3-ml amount of the cell suspension was mounted on a round cover slip (18 mm in diameter) which was placed in a 10-cm petri dish. Duplicate cover slips were used per virus dilution. After 20 hr of incubation at 36°C in a CO₂ incubator, cover slips were rinsed with phosphate-buffered saline (PBS), fixed with acetone, and stained with anti-NP fluorescent antibody. They were observed under a Tiyoda FM-200 A fluorescent microscope. Usually the number of fluorescent cells among 500 to 1,000 cells per cover slip was determined. When the proportion of fluorescent cells was lower than 1%, all fluorescent cells on a cover slip were counted. The NP antigen-forming unit (NPFU) of a given virus sample was calculated from the dilution at which between 1 and 10% of cells contained NP antigen. Preliminary experiments showed that an incubation time of 20 hr was sufficient for the NP antigen within an infected cell to reach a detectable level and that neither loss of infected cells due to sloughing-off into the medium nor an increase of uninfected cells by cell division occurred during this period.

UV-irradiation of the virus. Freshly harvested allantoic fluid from virus-infected chick embryos was employed. After the addition of phosphate buffer (pH 7.3) and bovine albumin at final concentrations of 0.5 M and 1%, respectively, the virus was dialyzed against PBS overnight at 4°C. The virus was distributed in 5-ml amounts in 10-cm petri dishes and was irradiated by a 15-w germicidal lamp at a distance of 20 cm for various time periods during which the open dishes were constantly rocked. The number of UV hits per virus particle, r , was calculated from the following equation: $V/V_0 = e^{-r}$. V_0 and V are virus titers before and after UV irradiation, respectively; e is the base of the natural logarithm.

RESULTS

Rescue of FL-marker of X-3311 virus by superinfection with CAM virus. We attempted to rescue the FL-marker of UV-inactivated X-3311 virus by superinfection with active CAM virus which lacks FL-marker, to determine whether the inactivation of FL-marker is dissociable from that of infectivity and, if so, to compare the inactivation rates of the two attributes. For this purpose, the occurrence of multiplicity reactivation should be avoided. At the same time, every FL-marker which has survived UV inactivation should be allowed to be rescued by superinfecting CAM virus. These requirements are met by making the MOI of UV-inactivated virus as small as possible to minimize the chance of multiple infection and by making the MOI of the superinfecting CAM virus high enough to ensure that all cells receive at least one reactivating virus particle. Clone 1-5C-4 cells were first infected with X-3311 virus, either active or UV-irradiated for different periods, at an MOI of 0.07 PFU/cell, based on preirradiation titer. One group of cells was superinfected with active CAM virus at an MOI of 3 PFU/cell, and the other group was treated with the maintenance medium in a similar way. An MOI of the superinfecting virus higher than 3 PFU/cell caused a slight decrease in plaque-forming infective centers. The cells, either singly or mixedly infected, were then plated on fresh FL monolayer cultures and plaque-forming infective centers were scored. The representative results are shown in Table 1 and Fig. 2. Infective centers formed by X-3311 virus alone, representing its surviving infectivity, decreased exponentially with the increasing UV dose. Superinfection with CAM virus caused an increase in the number of infective centers in different degrees, depending upon UV dose. Since the superinfecting CAM virus does not fully replicate in FL cells, additional plaque-forming infective centers must have resulted from the rescue of FL-marker of the inactivated X-3311 virus. Table 1 shows that cells infected even with nonirradiated X-3311 virus produced additional

TABLE 1. Reactivation of FL-marker of ultraviolet-inactivated X-3311 virus by superinfection with active CAM virus in clone 1-5C-4 cells^a

Time X-3311 virus ^b irradiated (sec)	No. of infective centers per 10 ⁶ cells plated			
	Not superinfected with CAM ^c		Superinfected with CAM ^c	
None	68,700	(100)	151,200	(100)
10	14,080	(20.5)	87,500	(57.9)
20	2,000	(2.91)	78,200	(51.7)
30	332	(0.48)	28,180	(18.6)
40	42.5	(0.062)	19,700	(13.0)
50			13,200	(8.75)
60			4,060	(2.49)

^a Singly or mixedly infected cells were plated on FL monolayers and plaque-forming infective centers were scored.

^b MOI was 0.07 PFU/cell (calculated from pre-irradiation virus titer).

^c MOI was 3 PFU/cell. Values in parentheses indicate per cent.

FIG. 2. Reactivation of FL-marker of UV-irradiated X-3311 by superinfection with active CAM in clone 1-5C-4 cells. The results presented in Table 1 are graphically shown here. Line A shows the decrease in infectivity and line B shows the decrease of reactivability of the FL-marker during irradiation. The right ordinate is the number of lethal UV hits per virus particle.

infective centers after superinfection. The increase ranging from 1.5- to 3-fold was consistently observed. This finding indicates the presence of virus particles that are not infectious by themselves but still retain intact gene(s) coding for the FL-marker. The number of infective centers resulting from marker rescue decreased also exponentially but much more gradually than infectivity during irradiation. During irradiation for 40 sec, for instance, the virus received 7.4 hits per particle, whereas the gene coding for the FL-marker re-

ceived only 2.0 hits (Fig. 2). The latter gene, therefore, has a smaller target size than the whole viral genome.

We next examined whether CAM virus can rescue the FL-marker in FL cells in which it does not fully replicate. FL cells, instead of clone 1-5C-4 cells, were mixedly infected and plated on FL monolayers by the above procedure. Inactivation rate of FL-marker was identical to that in the preceding experiment. Thus CAM virus rescued the FL-marker in a restrictive host cell as efficiently as in a permissive clone 1-5C-4 cell. This suggests that genes of CAM virus, other than the portion(s) corresponding to the FL-marker in X-3311 virus, are expressed normally in FL cells.

Inactivation of the ability to produce NP antigen by UV irradiation. We next investigated whether the gene which has survived UV radiation can be expressed in the cell infected with inactivated virus without the aid of superinfecting virus. For this purpose, we tried to find out whether there exists a virus particle that has lost its infectivity but still retains the capability to direct the synthesis of NP antigen by testing nonirradiated and irradiated virus samples for PFU and NPFU. Nonirradiated X-3311 virus contained approximately equal numbers of PFU and NPFU. One virus sample, for instance, had 5.3×10^8 PFU/ml and 4.8×10^8 NPFU/ml. Inactivation of infectivity proceeded again exponentially (Fig. 3). Irradiation

FIG. 3. Inactivation of infectivity and NP antigen-forming ability of X-3311 virus by UV irradiation. Infectivity was determined by titration of infective centers as described. NPFU was determined by infection of suspended FL cells with the virus at an MOI of 0.01 PFU/cell and enumeration of cells containing NP antigen. The right ordinate is the number of lethal hits per virus particle. Symbols: ●, PFU; ○, NPFU.

for 30 sec corresponded to about 6 hits per virus particle in this experiment. NPFU of nonirradiated virus samples was determined after infection of FL cells at an MOI of 0.01 PFU/cell, based on preirradiation titer. The low MOI employed virtually excluded the possibility of multiplicity reactivation. The inactivation rate of NPFU was indistinguishable from that of PFU (Fig. 3), indicating that only infectious virus directs the synthesis of NP antigen.

DISCUSSION

The inactivation of the FL-marker by UV radiation proceeds at a significantly slower rate than that of infectivity. If it is assumed that the rescue of the intact gene by superinfecting virus occurs with high efficiency and that the relative inactivation rates are an indication of functional genome size, then the portion of the genome of X-3311 virus coding for the FL-marker should not exceed one-third of the total ribonucleic acid. The FL-marker refers to the property of X-3311 virus which enables it to produce mature infectious progeny in FL cells and which is not shared by most influenza viruses including CAM virus. The latter virus, replication of which does not reach the stage of production of infectious virus, at least directs the synthesis of NP antigen and hemagglutinin in FL cells and causes cytopathic effects (6). There is also an indication that CAM virus-specific ribonucleic acid is produced in FL cells (6). Therefore, the small target size of the gene(s) governing the FL-marker is not unexpected. The FL-marker might be related to some processes involved in maturation or assembly of viral components. Simpson and Hirst (3) and Tumova and Pereira (8) utilized a similar genetic marker for their study of genetic recombination. Both groups infected chick embryo cells with a plaque-forming influenza virus that had been inactivated by UV, together with an active non-plaque-forming virus and obtained evidence for the rescue of the plaque-forming capability of the inactive parent. Although it is not known whether the plaque-forming ability of their viruses is analogous to the FL-marker in this study, the difference in host range offers a convenient marker for genetic studies.

In contrast to the dissociation of the inactivation of FL-marker from that of the infectivity, it was not possible to demonstrate the presence of virus particles that had lost infectivity by UV damage but were still capable of directing NP antigen synthesis. It is suspected, therefore, that damage inflicted by UV radiation upon the viral genome prevents the expression of the gene that has survived radiation, unless the remaining portions of the genome are provided by co-infecting virus. This is in marked contrast to the stepwise inactivation of the viral genome, which was observed in fowl plaque virus inactivated by Bayer A 139 (2). The reason for the discrepancy between UV-inactivated and chemically inactivated viruses is not known. RNA rendered noninfectious by cleavage of the phosphate backbone by Bayer A 139 might behave differently from UV-inactivated genome containing pyrimidine dimers.

ACKNOWLEDGMENTS

We thank C. Enomoto for her technical assistance and Edwin D. Kilbourne for reviewing the manuscript.

LITERATURE CITED

1. Barry, R. D. 1961. The multiplication of influenza virus. II. Multiplicity reactivation of ultraviolet irradiated virus. *Virology* 14:398-405.
2. Rott, R., and C. Scholtissek. 1964. Behavior of virus-specific activities in tissue cultures infected with myxoviruses after chemical changes of the viral ribonucleic acid. *Virology* 22: 169-176.
3. Simpson, R. W., and G. K. Hirst. 1961. Genetic recombination among influenza viruses. I. Cross reactivation of plaque-forming capacity as a method for selecting recombinants from the progeny of crosses between influenza A strains. *Virology* 15:436-451.
4. Sugiura, A., and E. D. Kilbourne. 1965. Genetic studies of influenza viruses. II. Plaque formation by influenza viruses in a clone of a variant human heteroploid cell line. *Virology* 26:478-488.
5. Sugiura, A., and E. D. Kilbourne. 1966. Genetic studies of influenza viruses. III. Production of plaque-type recombinants with Ao and Al strains. *Virology* 29:84-91.
6. Sugiura, A., M. Ueda, and C. Enomoto. 1969. The effect of actinomycin D on the synthesis of influenza virus-specific RNA. *Arch. Gesamte Virusforsch.* 26:105-117.
7. Tateno, I., S. Suzuki, A. Kawamura, H. Kawashima, N. Kusano, Y. Aoyama, A. Sugiura, Y. Akao, K. Oikawa, N. Homma, and M. Naito. 1962. Diagnosis of influenza by means of fluorescent antibody technique. *Jap. J. Exp. Med.* 32:531-559.
8. Tumova, B., and H. G. Pereira. 1965. Genetic interaction between influenza A viruses of human and animal origin. *Virology* 27:253-261.