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Multiplicity Reactivation of Reovirus Particles After Exposure to Ultraviolet Light

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ABSTRACT

McCLAIN, MARY E. (California State Department of Public Health, Berkeley), AND REX S. SPENDLOVE. Multiplicity reactivation of reovirus particles after exposure to ultraviolet light. J. Bacteriol. 92:1422–1429. 1966.—Exposure of reovirus suspensions to moderate doses of ultraviolet light results in essentially exponential inactivation of infectivity to survivals of 10^{-2} to 10^{-3}. With suspensions of sufficiently high particle concentration, larger doses of ultraviolet light (6 to 12 min) are associated with multiplicity reactivation (MR) which is demonstrable both by immunofluorescent-cell count and by plaque assay in FL human amnion cells. Similar effects are produced by photodynamic inactivation in the presence of proflavine, but not by thermal inactivation at 50 C. All three reovirus types exhibit MR under appropriate conditions, and all three interact in mixed ultraviolet suspensions with high efficiency. Progeny from FL cells infected under conditions of MR were as infectious as those of unirradiated inocula, with yields per cell ranging from 10^4 to 4×10^4 infective units.

Since the original observation by Luria (11) of multiplicity reactivation (MR) among the T-even group of bacterial viruses, and the subsequent theoretical development of the phenomenon by Luria and Dulbecco (12), demonstrations of similar effects with animal viruses have been relatively few. Among the ribonucleic acid (RNA) viruses, those of poliomyelitis (5), influenza (2), fowl plaque (17), and Newcastle disease (6, 9) have been shown to exhibit MR. With the deoxyribonucleic acid (DNA) animal viruses, MR has been reported only for the poxvirus group (1, 8). It is of interest that genetic recombination has so far been demonstrated with these same viruses. It is reasonable to assume, as postulated for the bacteriophages, that, with animal viruses, the same basic mechanisms are involved in these two phenomena.

The present report presents evidence for the occurrence of multiplicity reactivation with a double-stranded RNA virus after exposure to ultraviolet (UV) light.

MATERIALS AND METHODS

Cells. The FL human amnion cell line was maintained and used for virus assay and propagation as previously described (20).

Viruses. Reoviruses type 1 (Lang), 2 (Erwin, States), and 3 (Willis) were used. Stock suspensions were pepared from FL human amnion cells harvested 66 to 96 hr after infection, and were frozen and thawed six times. For use in these experiments, strains Lang and States were partially purified by exposure to α-chymotrypsin and nucleases, extraction with Genetron, and dialysis against 0.01 M phosphate-buffered saline (PBS). Strains Erwin and Willis were used as crude cell harvest and were clarified by centrifugation and treated with chymotrypsin for enhancement of infectivity (21). All virus suspensions were stored at −20 C.

Infectivity tests. Two methods of virus assay were used: immunofluorescent-cell count (ICC; 20) and plaque assay. Titers were expressed as fluorescent cells per milliliter (FC/ml) or plaque-forming units per milliliter (PFU/ml).

Exposure to ultraviolet light. Source of radiation was a 15-w General Electric germicidal lamp emitting more than 95% of its radiation at 2.537 A wavelength. Energy flux, measured with a Latarjet dosimeter, at the surface of irradiated samples was 15 erg per mm^2 per sec. Purified virus suspensions were diluted 5-fold, and crude suspension was diluted 30-fold in PBS for ultraviolet (UV) exposure. Sample volumes of 2 to 2.5 ml in 60-mm petri dishes were exposed with magnetic stirring. Dose of UV is expressed in minutes of exposure.

RESULTS

Effect of UV irradiation on reovirus infectivity. Irradiation of reovirus suspensions with UV for increasing periods of time, followed by ICC assay of residual infectivity, produced the results summarized in Fig. 1 and in Tables 1 and 2. There was

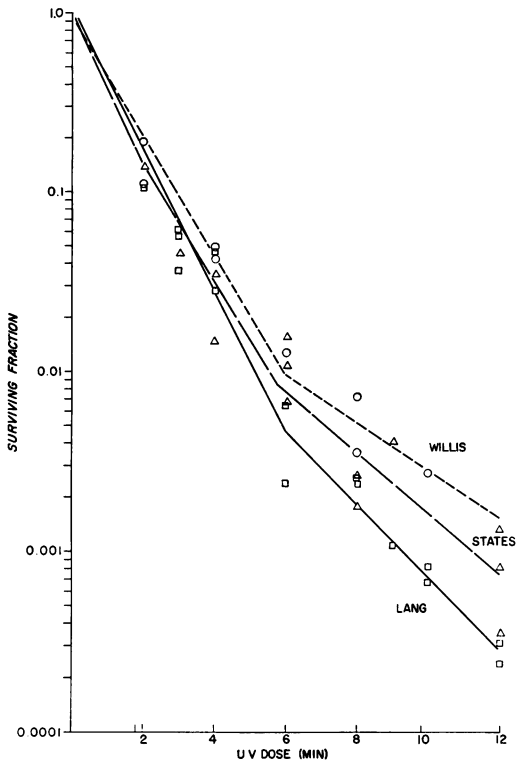

FIG. 1. *Inactivation of reoviruses by ultraviolet light. Symbols:* □ = *type 1;* △ = *type 2;* ○ = *type 3.*

a loss of infectivity for all three reovirus types which was essentially exponential with UV exposures of less than 6 min. This largest dose reduced survival to 10^{-2} to 10^{-3}. Type 1 (Lang) was slightly but consistently more sensitive to inactivation than strains of types 2 or 3, but the overall resistance to UV inactivation of all three reovirus types was considerably greater than reported for a number of other RNA viruses (13). With UV doses greater than 6 min, there was a decrease in the rate of inactivation. A precise estimate of residual infectivity was, however, complicated by effects suggestive of multiplicity reactivation.

Tables 1 and 2 summarize representative experimental data from infectivity assays of reoviruses type 1, 2, and 3 after exposure to UV, and also illustrate the type of effect encountered with larger UV doses. In titrations of unirradiated reovirus by the ICC method, there is a close linear relationship between the dilution of virus suspension inoculated and the number of fluorescent cells observable 20 to 24 hr later. This linearity has been shown to extend over a range of 10 to 2,000 fluorescent cells per 15 mm of cover slip culture with a variety of assay cells and several different reovirus strains (McClain, Spendlove, and Lennette, *in press*). In replicate assays, the experimental error of this method has been observed to approximate ±25%. Thus, the ratio between the observed number of fluorescent cells and the number expected in a particular dilution series would be 1.0 under ideal conditions but, in fact, ranges from 0.75 to 1.25, because of experimental fluctuations. With UV doses of 2 to 3 min, the expected linear relationship is still seen, and the ratios between observed and expected counts (O/E) ranged from 0.9 to 1.2. With 6- and 12-min exposures, the fluorescent-cell count at the highest dilution was used to obtain an estimate of the number of infected cells expected in lower dilutions of the same assay. Such calculations resulted in O/E ratios of 2.7 for the 6-min UV dose, and 31 for the 12-min exposure (Table 1). Comparable results were obtained with types 2 and 3 viruses with use of purified and crude suspensions (Table 2).

One of the essential conditions for MR is that it occurs only where susceptible cells have adsorbed at least two particles, one or both of which are inactive in single infection. The data in Tables 1 and 2 can be examined from this point of view.

TABLE 1. *Effect of ultraviolet irradiation (UV) on infectivity of reovirus type 1 (Lang) assayed by immunofluorescent-cell count*

UV dose	Dilution ($\log_{10}$)	FC count[a]		Observed/ expected
		Observed	Expected	
min				
0	4.5	575	570	1.0
	5.0	193	183	1.1
	5.5	57	57	1.0
3	3.5	420	360	1.2
	4.0	108	119	0.9
	4.5	36	36	1.0
6	3.0	133	50	2.7
	3.5	13	16	0.8
	4.0	5	5	1.0
9	2.5	410	40	10
	3.0	31	13	2.4
	3.5	4	4	1.0
12	2.0	1,570	50	31
	2.5	52	16	3.3
	3.0	5	5	1.0

[a] Observed FC count = average number of fluorescent cells counted on three FL cell cover slip cultures per dilution. Expected FC count is calculated from observed average at highest dilution tested at the corresponding UV dose.

TABLE 2. *Effect of ultraviolet irradiation on infectivity of reovirus types 2 and 3 measured by ICC test*

UV dose	Type 2 (States)				Type 3 (Willis)			
	Dilution (log_{10})	FC count[a]		O/E	Dilution (log_{10})	FC count		O/E
		Observed	Expected			Observed	Expected	
min								
0	4.5	133	120	1.1	3.0	382	410	0.9
	5.0	40	38	1.1	3.5	136	131	1.0
	5.5	12	12	1.0	4.0	41	41	1.1
2	3.5	348	310	1.1	2.5	216	250	0.9
	4.0	93	99	0.9	3.0	87	80	1.1
	4.5	31	31	1.0	3.5	25	25	1.0
4	3.0	200	150	1.3	2.0	310	200	1.5
	3.5	62	48	1.3	2.5	61	64	1.0
	4.0	15	15	1.0	3.0	20	20	1.0
6	2.5	300	90	3.3	1.5	622	260	2.4
	3.0	47	29	1.6	2.0	150	83	1.8
	3.5	9	9	1.0	2.5	26	26	1.0
8	2.5	83	30	2.8	1.0	2,000	280	7.2
	3.0	10	10	1.0	1.5	190	90	2.1
	3.5	3	3	1.0	2.0	28	28	1.0
10	2.0	930	50	19	1.0	700	130	5.4
	2.5	72	16	4.5	1.5	84	42	2.0
	3.0	5	5	1.0	2.0	13	13	1.0

[a] Observed FC count = average number of fluorescent cells counted on three FL cell cover slip cultures per dilution. Expected FC count is calculated from observed average at highest dilution tested with the corresponding UV dose.

The infectivity titer of the unirradiated Lang virus provides a minimal estimate of the number of originally functional particles adsorbed. Knowing the number of cells per cover slip (4 to 5 $\times$ 10^5) and using the Poisson equation, one can calculate that, at a 1:400 dilution, the adsorbed particle input would be approximately 0.1, and that 0.4% of cells could be expected to have adsorbed at least two or more particles. In the experiment shown in Table 1, the observed excess of infected cells inoculated with a 1:330 dilution ($10^{-2.5}$) of virus exposed to UV for 9 min was 370, representing approximately 0.1% of the total cells. Thus, it is evident that the particle multiplicity was adequate in this experiment to support the hypothesis of MR.

An apparent exception to the requirement is seen in the results with type 3 virus (Willis) shown in Table 2; they appear to indicate that the anomalous effects being considered as multiplicity reactivation could occur with inocula not expected to provide sufficient particle input. With an initial control titer of 3.8 $\times$ 10^5 FC/0.02 ml, an average multiplicity of 1 would be obtained with undiluted inocula, and one might expect that a 10^{-1} dilution would be the highest at which a significant excess of infective centers could be detected. In fact, evidence of MR was observed with the 10^{-2} dilution. Partial explanation of these results lies in the inefficiency of response of FL cells to infection with type 3 reoviruses, which leads to 5- to 50-fold underestimate of originally functional particle numbers. At this point, it is sufficient to note that results indicative of MR can be obtained in FL cells with all three reovirus types after exposure to UV.

Demonstration of MR by plaque assay. One limitation of the ICC method in verification of MR with UV suspensions is the difficulty of excluding the possibility that the observed excess of productive cells could be an artifact attributable to secondary cycles of infection. Incorporation of high-titer neutralizing antibody in the medium during ICC assay did not reduce the infected-cell counts in controls or abolish the MR effect in UV-exposed reovirus suspensions. Nevertheless, the most satisfactory method of excluding this potential source of error was the demonstration

of MR under conditions in which reinfection through the medium was restricted, as it is under an agar layer.

Unirradiated and UV-exposed type 1 reovirus was assayed simultaneously by ICC and plaque formation in FL cell cultures. Results of fluorescent-cell counts obtained at 20 hr and plaque counts at 7 days are summarized in Table 3. The control suspensions exhibited the expected correspondence between dilution and infective units in plaque assay as well as in ICC assay. With the irradiated suspension, an excess of productive infection at lower dilutions was also observed by both methods of assay. These results clearly demonstrate that the UV-induced multiplicity effects occur under conditions which preclude secondary foci of infection.

The plating efficiency of unirradiated type 1 reovirus by FL plaque assay in this experiment was approximately 0.2, compared with ICC titer. But the relative efficiency of plaque assay with UV-irradiated suspensions was considerably less even with inocula not inducing MR. With cells other than FL, the sensitivity of reovirus plaque assay compared with ICC can be increased to 1, but the differential in plating efficiency between control and irradiated reovirus persists (McClain, *in preparation*).

Multiplication of UV-exposed reoviruses. Multiplicity reactivation demonstrable by plaque formation implies development of infectious virus, but the relatively low efficiency observed could also signify that more than 90% of the UV particles which were functional in antigen formation detectable by fluorescence were incapable of producing fully infectious progeny. Such a result was observed by Barry (2), who found that progeny induced by MR among heavily irradiated influenza virus particles were completely noninfectious, although detectable by hemagglutination.

To clarify this point with reovirus MR, FL cover-slip cultures were inoculated with two 10-fold dilutions of unirradiated or UV-exposed suspensions of Lang, States, or Willis strains. Eighteen hours later, three cultures of each inoculum group were fixed and stained with fluorescent antibody to obtain a measure of the productive input. At the same time, parallel cultures in each group were harvested and assayed for infectivity. The capacity for multiplication of control or irradiated inocula was assessed by comparing the respective virus yield per fluorescent cell, an index obtained by dividing the total infectivity per cover slip culture by the number of fluorescent cells observed in parallel cultures. Results are summarized in Table 4.

The virus yield per fluorescent cell 18 hr after inoculation was extremely high for all three reovirus types, whether infection had been initiated by unirradiated virus or by two or more UV-damaged particles under conditions where MR was observed. The cell-associated infectivity ranged from 10^4 to 4×10^4 infective units per cell. The apparently lower yield of type 3 virus is a reflection of FL cell insensitivity to this reovirus type, as previously mentioned. The overall result

TABLE 3. *Comparison of MR effect with UV-irradiated type 1 reovirus by ICC and plaque assay*

UV dose	Dilution ($\log_{10}$)	ICC assay[a]		Plaque assay[a]	
		FC/0.02 ml	O/E	PFU/0.1 ml	O/E
min					
0	4.5	265	1.1		
	5.0	79	1.0	89	1.3
	5.5	24	1.0	27	1.2
	6.0			7	1.0
10	1.5			≦300[b]	≦10
	2.0	1,050	8.8	30	3.0
	2.5	48	1.2	3	1.0
	3.0	12	1.0	1	1.0

[a] Average number of infective units (fluorescent cells or plaques) in three cultures per dilution. Observed-expected ratio is derived as in Table 1.
[b] Partial confluence.

TABLE 4. *Virus yields from FL cells 18 hr after infection with unirradiated or UV-exposed reoviruses*

UV dose	Inocula			18-hr Virus yields	
	Strain	Dilution ($\log_{10}$)	FC count[a]	Infectivity/cover slip	Infectivity/FC (10^8)
min					
0	Lang	3.5	1,400	3.2×10^7	23
		4.5	110	1.3×10^6	12
	States	4.5	850	3.6×10^7	42
		5.5	68	3.0×10^6	44
	Willis	3.0	130	1.1×10^6	8.5
		4.0	15	8.3×10^4	5.5
10	Lang	2.0	580	2.2×10^7	37
		3.0	8	6.0×10^4	8
	States	2.5	800	1.7×10^7	21
		3.5	7	1.8×10^5	26
	Willis	1.0	129	1.7×10^6	13
		1.5	15	4.9×10^4	3.3

[a] Average fluorescent-cell count of three cover-slip cultures.

of this experiment indicates that the amount of infectious virus produced in reovirus MR is comparable to that of unirradiated particles during single-cycle growth in FL cells.

Effect of photodynamic and thermal inactivation on MR. The principal site of UV damage in DNA viral particles is generally considered to be the nucleic acid molecule through the formation of thymine dimers. Although precise definition of the UV lesion responsible for loss of biological activity in single- or double-stranded RNA remains to be made, there is little doubt that nucleic acid is damaged. The site of photodynamic inactivation in the presence of heterocyclic dyes is also considered to be nucleic acid, whereas thermal inactivation is presumed to act primarily through alteration of viral proteins. It was of interest to determine whether MR would be evident in reovirus suspensions inactivated by these means.

Suspensions of reoviruses in PBS were mixed with proflavine to yield a dye concentration of 1.5 mg/ml. Control and dye-virus mixtures were held in the dark at 4 C overnight. All suspensions were then diluted 10-fold with PBS and were exposed to two 15-w fluorescent lights at a distance of 10 cm for 6 min. Results of infectivity assays are shown in Table 5.

Reoviruses types 1 and 2 differed markedly in their photosensitivity in the presence of proflavine, with type 1 being approximately 10 times more sensitive than the two type 2 strains, crude or purified. Clear evidence of MR was seen with all four suspensions after photodynamic inactivation, although the extent of MR demonstrable with the type 2 strains was comparatively small, because of higher residual infectivity.

No evidence of MR was observed with any of the three reovirus types inactivated by heating at 50 C to survival in the range of 10^{-3} to 10^{-6}. As with photodynamic inactivation, the three types differed in sensitivity to thermal inactivation, Lang being considerably more thermolabile than States or Willis. Type 1 inactivation at 50 C was several orders of magnitude greater than that reported by Wallis et al. (21), reflecting the en-

TABLE 5. *Multiplicity reactivation with reoviruses photodynamically inactivated in the presence of proflavine*

Reovirus type	Strain	Treatment	Dilution ($\log_{10}$)	FC count[a]		O/E	Survival
				Observed	Expected		
1	Lang, purified	Control	5.5	180	214	0.8	1.0
			6.0	67	67	1.0	
		Proflavine	3.0	610	290	2.1	4.3×10^{-3}
			3.5	102	93	1.1	
			4.0	29	29	1.0	
	Lang, crude	Control	4.5	262	246	1.1	1.0
			5.0	77	77	1.0	
		Proflavine	2.0	180	50	3.6	9.6×10^{-4}
			2.5	23	16	1.4	
			3.0	5	5	1.0	
2	States, purified	Control	6.0	183	195	0.9	1.0
			6.5	61	61	1.0	
		Proflavine	4.5	133	90	1.5	1.4×10^{-2}
			5.0	43	33	1.3	
			5.5	9	9	1.0	
	Erwin, crude	Control	4.0	320	350	0.9	1.0
			4.5	110	110	1.0	
		Proflavine					
			2.5	610	320	1.9	3×10^{-1}
			3.0	190	105	1.8	
			3.5	32	32	1.0	

[a] Observed FC count = average number of fluorescent cells counted on three FL cell cover slip cultures per dilution. Expected FC count as derived in Table 1.

hanced susceptibility to thermal inactivation after chymotrypsin treatment previously noted by Spendlove and Schaffer (21).

Interaction between reovirus types after UV inactivation. The three reovirus types are distinguishable by both biological and immunological criteria, but much evidence also exists for a close relationship between types (14). Many of the observed similarities in antigenicity, hemagglutination, reactivity to enzymes, or surface-active agents may be ascribed to capsid proteins rather than directly to the nucleic acid. Consequently, it was of interest to determine whether UV-damaged reovirus particles of different antigenic types were capable of interacting in MR.

Suspension of UV-exposed types 1 and 2 were diluted to a point above and below that at which MR had previously been shown, and each dilution was then mixed with an equal volume of diluent or of the second virus. Controls and mixtures were then assayed in FL cover slip cultures. In the absence of interaction, it was expected that the infected-cell count produced by a given mixed inoculum would be equivalent to the sum of the two separate controls.

Results are shown in Table 6. In fluorescent-cell counts of the two separate control series, the lowest dilution showed MR, whereas the two higher dilutions gave numbers expected with a twofold dilution. Seven of the nine mixtures produced counts significantly greater than the sum of the constituent controls, the excess averaging slightly more than 100%. In other experiments of similar design, it was established that each of the three types was capable of interaction in MR with heterologous strains with an efficiency indistinguishable from that of homologous particles. In contrast, heat-inactivated particles could not be shown to participate in MR with UV-exposed particles. Identification of progeny from interaction of heterologous UV particles is under investigation.

TABLE 6. *Multiplicity reactivation between type 1 (Lang) and type 2 (States) reoviruses after ultraviolet irradiation*[a]

FC count in controls		FC count in mixtures					
Lang	States	Avg count[b]	Ratio[c]	Avg count[b]	Ratio[c]	Avg count[b]	Ratio[c]
36	200	575/236	**2.4**	123/72	**1.7**	66/54	**1.2**
10	36	342/210	**1.6**	110/46	**2.4**	67/28	**2.4**
5	18	215/205	**1.0**	110/41	**2.7**	40/23	**1.7**

[a] Suspensions of each UV-exposed virus were diluted in twofold steps. Each dilution was then mixed with an equal volume of diluent or with the three dilutions of the second virus. Single controls and mixtures were each assayed in three FL coverslips.

[b] Numerators represent average count of fluorescent cells observed in mixed inocula; denominators are sum of average FC counts of separate controls for each mixture.

[c] Number in boldface type is ratio of observed to sum of separate controls.

DISCUSSION

Recognition of multiplicity reactivation with reovirus, as with other viruses, is dependent upon having a sufficient concentration of particles so that multiplicities greater than 1 can be attained in a significant number of indicator cells after appropriate inactivation. In certain systems, this requisite multiplicity has been achieved by particle clumping with $MgCl_2$, a procedure used effectively with vaccinia virus (1) and Newcastle disease virus (9). Particle clumping was not necessary for demonstration of reovirus MR, nor did particle aggregation contribute in any significant degree to the effects which were observed. Sonic treatment of purified suspensions used in these MR experiments neither altered the infectivity of controls nor reduced the degree of MR obtained, although infectivity of suspensions not treated with chymotrypsin was increased three- to eightfold after sonic treatment. Where residual infectivity is measured by plaque assay, the size of plaques limits the number of infective units which can be detected without introduction of overlap bias (10), and thus restricts the range of quantitation within which MR may be recognized without excessive numbers of experimental cultures. These two factors, low particle multiplicity and limitations of assay systems, probably account for the fact that MR was not observed in the investigation of UV sensitivity of type 3 reovirus (13) and of photodynamic inactivation with type 1 virus (23). Recognition of reovirus MR is facilitated by use of immunofluorescence assay, primarily by the comparatively large numbers of infective units which can be detected in a small cell population.

In addition to MR, there are data in the present study and in experiments to be reported later which suggest involvement of two other types of phenomena. The first of these is a functional heterogeneity among UV-exposed reovirus particles which is revealed in the reduced plating efficiency of such suspensions by plaque as com-

pared with ICC assay (Table 3). The relative efficiency of plaque formation dropped from 0.2, with unirradiated type 1 virus, to less than 0.01, even with inocula in which MR was not observed in either system. This effect has been observed with strains of the other two types and in cells other than FL, and thus is not an attribute of a specific virus-cell interaction. It suggests that there are at least three types of particles in UV reovirus suspensions; those which are functional in multiparticle infection measured both by plaque formation and by immunofluorescence, those with minimal or no UV damage, which are functional in both systems as single particles, and a third class, which is defective in plaque formation, but is functional in a single cycle of infection.

The second phenomenon alluded to above is seen in a difference in slope of UV survival curves determined in different cell lines. These results simulate those of Ellison et al. (7) and suggest by analogy the possibility of host-cell repair mechanisms. Unirradiated reovirus plates with nearly equivalent efficiency in both BS-C-1 and L929 cells. After UV exposure, there is a progressive decrease in plating efficiency in L929 cells to less than 0.1 of that of BS-C-1, both in plaque and ICC assay. It is tempting to speculate that this difference reflects a capacity of the latter cells for at least partial repair of the UV damange in RNA molecules, which L929 cells lack. Preliminary attempts to damage this hypothetical repair system by UV have not been successful, but this treatment would not be expected to affect the activity of enzymes pre-existing in the cytoplasm where reoviruses replicate.

In view of the number of variables which appear to be involved in reovirus MR and which remain to be investigated systematically, it has not seemed appropriate to undertake more than limited quantitative analysis of the results in the present report. Particle counts of the purified Lang and States suspensions were consistent with a particle-infectious unit ratio less than 10 and close to 1, comparable to the ratio of 2.5 reported for a type 1 reovirus by Wallis et al. (22). Correlation of numbers of productive cells observed with the number expected to have adsorbed two or more UV-exposed particles indicates comparatively efficient interaction in MR with low UV doses. Furthermore, all three reovirus types interact with equivalent efficiency in UV-induced MR. A variety of mutants has been isolated which may give some insight into the basic mechanisms of reovirus MR. Demonstration of recombination under conditions of MR would clearly establish the genetic basis of the MR interaction, a result which may be anticipated in view of the observation of genetic recombination in NDV under similar conditions (9).

The identity of the UV lesion in either single- or double-strand RNA molecules remains to be determined, and so do the mechanisms by which such lesions are repaired to yield functional genomes. Considerable progress has been achieved in identifying the formation of thymine dimers as the primary molecular lesion in DNA and in correlating their presence with loss of biological activity (3, 18). Recognition of the reversibility of thymine dimerization by photoenzymatic processes (15, 16) and the existence of excision mechanisms (4, 19) has elucidated the processes by which UV-induced lesions are repaired in DNA molecules. Multiplicity reactivation by definition involves some type of repair. The fact that it can be demonstrated so clearly with reoviruses, unique among RNA animal viruses in their double-stranded character, should recommend them as a model system for similar investigations with RNA molecules.

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