Studies of Base-Sequence Homology Among Some Cytoplasmic Deoxyriboviruses of Vertebrate and Invertebrate Animals

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Relationships among some cytoplasmic deoxyriboviruses were investigated by deoxyribonucleic acid/ribonucleic acid hybridization experiments. Evidence of base-sequence homology between nucleic acids of different viruses was found within, but not between, the poxvirus and iridescent virus groups. Viruses of the vaccinia subgroup of poxviruses were 30 to 100% related, and the degree of relatedness was correlated with serological relatedness. Relationships within the iridescent virus group depended on the laboratory of origin and passage history of the virus strains used. None of these viruses was related to a superficially similar virus of frogs.

It was proposed in previous reports that members of certain groups of viruses have similar properties because their nucleic acids have common base sequences (2-4). We report here the results of experiments designed to test hypotheses of base-sequence homology within, but not between, the poxvirus and iridescent virus groups, and the lack of homology between any of these viruses and frog virus 3, which superficially resembles iridescent viruses but differs from them in base composition (4, 15).

The degree of sequence homology between two genomes can best be estimated by the competitive hybridization test. This was not possible in the present work because the necessary quantities of viral nucleic acids were not available. A similar restriction on the availability of radioactively labeled nucleic acids prevented the estimation of homology by saturation curves. The technique used was hybridization between viral deoxyribonucleic acid (DNA) and radioactive ribonucleic acid (RNA) synthesized in vitro with viral DNA as primer (16). The experimental design was based on that used in the taxonomy of mycoplasmas (21, 22, 25, 26). Somerson et al. (25) showed that, under conditions of DNA excess, the ratio of synthetic RNA bound by a given DNA to that bound by the type of DNA used to prime the polymerase reaction was constant over a wide range of DNA and RNA concentrations and could be taken as a measure of the relatedness of the two types of DNA. However, all sequences in viral DNA may not be transcribed in vitro with equal frequency (7, 13), so that quantitative estimates of the extent of homology may be biased.

Another difficulty with experiments of this type is that salt concentration and temperature may affect the result. Hybrids estimated in our experiments are those formed in 16 hr at 65 C in $6 \times \text{SSC}$ (0.90 M NaCl plus 0.09 M sodium citrate) and stable to 20 μg of ribonuclease per ml for 1 hr at room temperature, conditions found to be approximately optimal for specific formation of hybrids between DNA from *Sericesthis* iridescent virus and complementary RNA. It will be shown that hybrid formation under these conditions is highly specific. However, the results are quantitatively variable and the relatedness values reported for homologous viruses must be treated as preliminary approximations only.

MATERIALS AND METHODS

Glassware. All glassware was heated at 120 C for 24 hr to eliminate contamination with nucleases.

Filters. Sartorius Membranfilter type MF50 (25 mm; Sartorius Membranfilter GmbH, 24 Göttingen, Germany) were used in all experiments. Filters were autoclaved before use.

Solutions. SSC is 0.15 M NaCl and 0.015 M sodium citrate (pH 7); $0.001 \times \text{SSC}$, $0.01 \times \text{SSC}$, and $6 \times \text{SSC}$ are solutions containing 0.001, 0.01, and 6 times these concentrations, respectively. Solutions of heat-labile materials were made up in sterile solvents, all other solutions were autoclaved.

Enzymes. *Escherichia coli* RNA polymerase (specific activity, 3,500 units/mg) was purchased from Biopolymers Inc., Pinebrook, N.J., and stored at -20 C. Deoxyribonuclease (electrophoretically purified) and ribonuclease were from Worthington Biochemical Corp., and Pronase (B) was from Calbiochem. The

ribonuclease was heated to 90 C for 10 min at pH 5 before use.

Viruses. Poxviruses were grown (from cloned stock, when possible) in chick embryo cell cultures or on the chorioallantoic membrane of eggs, and were purified by the method of Joklik (17). The following strains were used: vaccinia, Mill Hill strain; neurovaccinia, Hall Institute and Pasteur Institute strains; rabbitpox, Utrecht and Rockefeller Institute strains; ectromelia, Moscow strain; cowpox, Brighton strain (9, 10).

Iridescent viruses were grown in larvae of the wax-moth *Galleria mellonella* by M. F. Day, N. Fukaya, A. J. Gibbs, and J. Mitsuhashi and in *Tipula paludosa* by C. F. Rivers, and were purified by the method of Day and Mercer (8).

Frog virus 3 (15), received from A. Granoff, was cloned and grown in chick embryo cells at 25 C. The infected cells were homogenized and the nuclei and cell debris were removed by centrifugation. Virus was purified by the method used for poxviruses (17), except that the pH of the buffer was 8, and the virus was twice centrifuged through 30% rather than 36% sucrose.

Preparation of DNA. Virus was suspended in SSC and, after incubation for 4 to 5 hr at 60 C with sodium dodecyl sulfate (2%) and Pronase (100 μ g/ml), the DNA released was extracted three times with phenol as described previously (5), precipitated with ethyl alcohol, and dissolved in $0.1 \times$ SSC. The concentration of DNA was estimated from the absorbance of the solution at 260 nm.

Synthesis and purification of radioactive complementary RNA. Radioactive RNA complementary to each DNA was synthesized from tritiated adenosine, guanosine, cytidine, and uridine 5' triphosphates (1 to 4c/mole, Schwarz Bio Research) by the method of Rose, Reich, and Weissman (23), with the following modifications. A 20-unit amount of RNA polymerase was used in each reaction, incubation was for 30 min, and, after the primer was removed with deoxyribonuclease, the mixture was incubated for 5 min more with 50 μ g of Pronase. The tritiated RNA was extracted once with sodium dodecyl sulphate, three times with phenol at 37 C (19), and once with ether; it was twice precipitated with ethyl alcohol and was stored in 70% ethyl alcohol at -20 C.

Formation and detection of DNA/RNA hybrids. The method of Gillespie and Spiegelman (14) was used, except that DNA was denatured by heating to 98 C for 10 min in $0.001 \times$ SSC and rapidly poured into a 30-fold excess of ice-cold $6 \times$ SSC. A solution containing 5 μ g of denatured DNA in 10 ml of $6 \times$ SSC was slowly passed through each filter, which retained about 95% of the DNA. The DNA/RNA ratios in hybridization experiments were never less than 100:1 as calculated from the specific activity of the RNA preparations.

Each radioactive RNA was reacted with each DNA, and the radioactivity bound was expressed as a fraction of that bound by an equal amount of primer DNA. The reciprocal experiment was also done, and the geometric mean of the two results was used as a relatedness statistic. This statistic allows for differences in the ability of the various DNA and RNA

preparations to form hybrids; it is comparable to the titer ratio used in immunology (1) and to the square root of the relatedness values of Reich et al. (21). Confidence intervals and tests of significance were based on analysis of variance by using the log-normal distribution.

The density gradient centrifugation method was described elsewhere (5).

RESULTS

Specificity of hybridization between DNA and complementary RNA synthesized in vitro. *Sericesthis* iridescent virus DNA was used to prime the synthesis of radioactive complementary RNA, which was then incubated with filters to which various DNA preparations had been attached. Specific, ribonuclease-resistant DNA/RNA hybrids were formed with DNA from *Sericesthis* iridescent and the serologically related *Tipula* iridescent viruses, but not with DNA from bacteria or vertebrates (Table 1). The noise level in terms of the counts per minute of RNA retained by a filter without DNA was 0.2% of the input, and nonspecific binding by unrelated DNA was 1 to 2% of that by the DNA used to prime the synthesis.

Sequence homology within but not between different virus groups. DNA preparations from four poxviruses and three iridescent viruses were used to prime the polymerase reaction, and each DNA was reacted with each RNA. DNA and synthetic RNA from frog virus 3 were also included.

All reactions within the poxvirus and iridescent

TABLE 1. *Specificity of hybridization*

DNA on filter ^a	<i>Sericesthis</i> iridescent virus-complementary RNA bound to filter
	counts/min
<i>Sericesthis</i> iridescent virus.....	1,664
Calf thymus.....	18
Chick embryo.....	20
<i>Escherichia coli</i>	14
<i>Micrococcus lysodeikticus</i>	24
None.....	17
<i>Sericesthis</i> iridescent virus.....	1,737
<i>Tipula</i> iridescent virus.....	1,278
Calf thymus.....	27

^a Each filter (5 μ g of DNA) was incubated for 16 hr at 65 C with approximately 10,200 counts/min of radioactive RNA (synthesized with *Sericesthis* iridescent virus DNA as primer) in 1 ml of $6 \times$ SSC containing 0.01% sodium dodecyl sulfate, and treated as described in Materials and Methods.

virus groups gave specific hybridization, but there were only slight reactions between nucleic acids of viruses from different groups, or between nucleic acid from frog virus 3 and any other virus (Table 2). Within-group relatedness values varied from about 30 to 100% (Table 3). Most between-group reactions indicated less than 1% relatedness, although occasional reactions up to 4% relatedness occurred.

Relationships within the poxvirus group. Relationships within the vaccinia subgroup of poxviruses in our experiments are similar to their serological relationships in complement-fixation tests (27); the closest relationship is between vaccinia and rabbitpox (89% related by nucleic acid

hybridization, 100% by complement fixation) and the most distant that between cowpox and ectromelia (28% related by nucleic acid hybridization, 13% by complement fixation). There are exceptions to this correlation; for instance, ectromelia and rabbitpox are 60 to 70% related by DNA/RNA hybridization, but only about 20% by complement fixation.

The experimental error of the method was such that viruses more than 60 to 65% related could not be distinguished with a probability of less than 0.05, although all relationships other than that between vaccinia and rabbitpox were close to the significance limit. The main source of error was the difference between reciprocal experiments.

TABLE 2. DNA/RNA hybridization among poxviruses, iridescent viruses, and frog virus 3^a

DNA on filter	DNA used to prime polymerase reaction								
	Rabbitpox	Vaccinia ^b	Vaccinia ^b	Cowpox	Ectromelia	Frog virus 3	<i>Sericesthis</i> iridescent ^c	<i>Tipula</i> iridescent ^c	<i>Chilo</i> iridescent ^d
Rabbitpox (Utrecht)...	453	94	6,656	289	1,125	<1 ^e	23	<1	<1
Vaccinia (Mill Hill)...	507	153	6,627	390	919	ND	146	39	ND
Cowpox.....	330	86	ND ^f	571	ND	ND	75	70	ND
Ectromelia, (Moscow).....	198	95	2,710	158	1,081	<1	2	<1	29
Frog virus 3.....	<1	<1	<1	<1	32	117	<1	13	70
<i>Sericesthis</i> iridescent ^e	<1	<1	77	<1	22	<1	2,231	1,666	15,730
<i>Tipula</i> iridescent ^e	<1	<1	<1	<1	<1	<1	2,062	1,926	5,218
<i>Chilo</i> iridescent ^d	<1	<1	<1	<1	<1	<1	2,306	2,303	9,594

^a Each result is the geometric mean of counts per minute of ³H-RNA bound per filter, less counts per minute bound to a control filter to which 5 µg of chick embryo DNA was attached.

^b Experiment repeated.

^c Present Canberra strains.

^d Virus from Mitsuhashi (1968).

^e Mean less than 1 count/min above the control.

^f Not done.

TABLE 3. Percentage relatedness of some poxviruses, iridescent viruses, and frog virus in hybridization experiments^a

Virus	Vaccinia (Mill Hill)	Cowpox	Ectromelia (Moscow)	Frog virus 3	<i>Sericesthis</i> iridescent	<i>Tipula</i> iridescent	<i>Chilo</i> iridescent
Rabbitpox (Utrecht)	89	62	68	<1	<1	<1	<1
Vaccinia (Mill Hill)		68	61	<1	<1	<1	<1
Cowpox			28	<1	4	4	<1
Ectromelia (Moscow)				<1	<1	<1	<1
Frog virus 3					<1	<1	<1
<i>Sericesthis</i> iridescent						89	130
<i>Tipula</i> iridescent							82

^a Relatedness values were calculated from the data of Table 2; each is the geometric mean of two values (three for vaccinia, one value only for relationships reciprocal to these indicated as not done in Table 4). The significance limit ($P < 0.05$) for distinguishing between DNA preparations is 60% relatedness.

There is no general agreement about the origin of rabbitpox virus. Although rabbitpox can be recognized as a "disease entity" (6, 20), there are reasons for regarding the viruses known as rabbitpox virus as derivative strains of neurovaccinia virus (11). Hybridization experiments with nucleic acids from one strain of vaccinia and two strains each of neurovaccinia and rabbitpox supported this view. All experiments resulted in the formation of specific DNA/RNA hybrids (Table 4). Relatedness values among these virus strains (72 to 100%, Table 5) were higher than those among other poxviruses studied (Table 3), and none differed from 100% with a probability less than 0.05. These virus strains are therefore so closely related, at least in those sequences transcribed *in vitro*, that they cannot be distinguished by a hybridization experiment of this type. This does not, of course, prove that the viruses are identical in all respects.

Relationships within the iridescent virus group. The three iridescent viruses appeared very closely related in DNA/RNA hybridization experiments; *Chilo* and *Sericesthis* iridescent viruses were apparently identical to each other, and more than 80% related to *Tipula* iridescent virus (Table 3), all these values falling within the 95% confidence limits for identical nucleic acids. A. J. Gibbs (*personal communication*) found the same strains of these viruses to be serologically indistinguishable. Although this does not prove that the viruses are identical, these results with recent laboratory strains conflict with earlier reports that the three viruses are serologically distinct and differ slightly in base composition (5, 8, 12).

It was originally reported that mixtures of DNA preparations from *Chilo* and *Sericesthis* iridescent viruses could be resolved into two partly overlapping bands in CsCl density gradients centrifuged at 44,700 rev/min for 24 hr (5). When this experiment was repeated with DNA from present laboratory strains, 100% related in DNA/RNA

hybridization experiments (Table 3), a single band was obtained (Fig. 1a). However, DNA preparations from samples of *Chilo* and *Sericesthis* iridescent viruses received from the laboratories of M. F. Day and N. Fukaya in 1965 and 1966 again gave a double band when centrifuged together in a CsCl gradient (Fig. 1b). The experiments were repeated, and again DNA preparations from early passage levels of the two viruses could be distinguished, but those from recent laboratory strains could not.

The relationships among iridescent viruses were therefore reinvestigated by DNA/RNA hybridization experiments with nucleic acid preparations from the earliest available passage levels of the three viruses. The nucleic acids of these viruses could readily be distinguished (Table 6); although specific annealing of nucleic acid from *Sericesthis* iridescent virus with that from either of the other viruses occurred, hybrid formation between nucleic acid preparations from *Chilo* and *Tipula* iridescent viruses was minimal. The relationship of these strains of *Chilo* and *Tipula* iridescent viruses can thus only be inferred from their common relationship to *Sericesthis* iridescent virus.

If present strains of iridescent viruses are virtually identical, but earlier passage levels differed, then one or two of the present strains must differ from earlier passage levels of the "same" virus. The present strains of *Chilo* and *Sericesthis* iridescent viruses were therefore compared with earlier passage levels of these viruses in DNA/RNA hybridization experiments.

Whereas the original and present strains of *Chilo* iridescent virus were indistinguishable, the present Canberra laboratory strain of *Sericesthis* iridescent virus was obviously different from the original virus; this difference was significant at the 0.1% level (Table 7). DNA from the present Canberra strain of "*Sericesthis*" iridescent virus could not be distinguished from that of *Chilo* iridescent virus, as found previously.

TABLE 4. Hybridization experiments with nucleic acids from vaccinia, neurovaccinia, and rabbitpox viruses^a

DNA on filter	DNA used to prime polymerase reaction				
	Vaccinia (Mill Hill)	Neurovaccinia (Hall Inst.)	Neurovaccinia (Pasteur Inst.)	Rabbitpox (Utrecht)	Rabbitpox (Rockefeller Inst.)
Vaccinia (Mill Hill).....	523	757	927	359	140
Neurovaccinia (Hall Inst.)....	943	2,437	2,520	1,065	365
Neurovaccinia (Pasteur Inst.)...	1,037	3,083	3,267	2,015	413
Rabbitpox (Utrecht).....	1,173	2,594	3,065	1,510	493
Rabbitpox (Rockefeller Inst.)...	644	1,261	1,221	574	296

^a Results expressed as counts per minute of ³H-RNA bound to filters.

TABLE 5. *Percentage relatedness of some strains of vaccinia and rabbitpox viruses in DNA/RNA hybridization experiments^a*

Virus	Neurovaccinia (Hall Inst.)	Neurovaccinia (Pasteur Inst.)	Rabbitpox (Utrecht)	Rabbitpox (Rockefeller Inst.)
Vaccinia (Mill Hill)	75	75	73	76
Neurovaccinia (Hall Inst.)		99	87	80
Neurovaccinia (Pasteur Inst.)			>100	72
Rabbitpox (Utrecht)				79

^a Relatedness values were calculated from the data in Table 4; each is the geometric mean of two values.

TABLE 6. *Differences among early-passage levels of iridescent viruses^a*

DNA on filter	DNA used to prime polymerase ^b		
	<i>Sericesthis</i> iridescent (Day 1965)	<i>Tipula</i> iridescent (Rivers 1965)	<i>Chilo</i> iridescent (Fukaya 1966)
<i>Sericesthis</i> iridescent (Day 1965)...	4,616	56	309
<i>Tipula</i> iridescent (Rivers 1965)....	224	2,201	11
<i>Chilo</i> iridescent (Fukaya 1966)....	449	7	1,830

^a Viruses used were the earliest passage levels available; the laboratory of origin and date of each virus are indicated.

^b Results are expressed as counts/min of ³H-RNA bound to filters.

DISCUSSION

Hybridization experiments between viral DNA and synthetic complementary RNA have confirmed the existence of homologous base sequences in nucleic acids of different poxviruses, and of similar homology between different iridescent viruses, although relationships among the original isolates of iridescent viruses are distant. No such homologies were found between the nucleic acids of viruses from different groups, or between frog virus 3 DNA and nucleic acid from any poxvirus or iridescent virus.

The relationships found in annealing experiments show a general correlation with serological relationships. However, quantitative interpretation of the results is limited by the likelihood of incomplete transcription of the DNA *in vitro* and by experimental variation which makes it difficult to distinguish between closely related nucleic acids. These difficulties could possibly be overcome by the use of nonenzymatic labeling methods (18,

FIG. 1. Microphotometer tracings of ultraviolet absorption photographs of mixtures of DNA preparations from *Chilo* and *Sericesthis* iridescent viruses at equilibrium in *CsCl* density gradients after 24 hr of centrifugation at 44,700 rev/min. The marker at a density of 1.731 g cm⁻³ is *Micrococcus lysodeikticus* DNA. (a) DNA from present laboratory strains of the viruses, 100% related in hybridization tests (Tables 2 and 3), formed a single band at a density of 1.689. (b) DNA from early-passage levels of the viruses, readily distinguishable by hybridization tests (Table 6), formed a double band with modes at 1.688 and 1.691 g cm⁻³.

24) and of competition experiments when larger samples are available.

A comparison of three members of the vaccinia subgroup of poxviruses, vaccinia, neurovaccinia, and rabbitpox viruses showed them to be more

TABLE 7. Relationships among strains of *Chilo* and *Sericesthis* iridescent viruses

DNA used to prime polymerase reaction	DNA on filter	³ H-RNA bound to filter
		counts/min
<i>Chilo</i> iridescent (Fukaya, 1966)	<i>Chilo</i> iridescent (Fukaya, 1966)	965
		1,179
	<i>Chilo</i> iridescent (Canberra strain, 1968)	1,618
		2,112
<i>Sericesthis</i> iridescent (Day, 1965)	" <i>Sericesthis</i> " iridescent (Canberra strain, 1968)	1,393
	<i>Sericesthis</i> iridescent (Day, 1965)	2,923
		3,454
		3,049
	" <i>Sericesthis</i> " iridescent (Canberra strain, 1968)	427
		446

closely related to each other than to cowpox and ectromelia viruses and to be indistinguishable by the technique used here. This lends support to the view that rabbitpox viruses are strains of neurovaccinia which have become epizootic in certain laboratory colonies of rabbits.

The clear evidence, that results with *Sericesthis* iridescent virus depend on the laboratory of origin and passage history of the virus strain used, suggests that there is an urgent need for an extension of the type culture collection and reference laboratory system to an international service to include all viruses. Differences of this magnitude between viruses given the same name could easily cause unnecessary and unproductive controversy.

It is probably not possible to reconstruct the precise history of the present Canberra laboratory strains of iridescent viruses. It appears that the present Canberra strain of "*Sericesthis*" iridescent virus is *Chilo* iridescent virus, although it may consist of a mixture with *Chilo* iridescent virus as the main component. It is doubtful whether this replacement of one virus by related virus could have been detected by any other technique.

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