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Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide

(tridecamer deoxyribonucleotide/hybridization competitor/hybridon)

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ABSTRACT **The tridecamer d(A-A-T-G-G-T-A-A-A-A-T-G-G), which is complementary to 13 nucleotides of the 3′- and 5′-reiterated terminal sequences of Rous sarcoma virus 35S RNA, was added to chick embryo fibroblast tissue cultures infected with Rous sarcoma virus. Inhibition of virus production resulted. The inference emerges that the tridecamer and its counterpart with blocked 3′- and 5′-hydroxyl termini enter the chick fibroblast cells, hybridize with the terminal reiterated sequences at the 3′ and 5′ ends of the 35S RNA, and interfere with one or more steps involved in viral production and cell transformation. Likely sites of action are (*i*) the circularization step of the proviral DNA intermediate, and (*ii*) the initiation of translation, the latter being described in the following communication [Stephenson, M. L. & Zamecnik, P. C. (1978) *Proc. Natl. Acad. Sci. USA* 75, 285–288].**

The primary structure of Rous sarcoma virus (RSV) 35S RNA has recently been determined at the 3′ (1) and 5′ ends (2) by two groups working independently. A 21-nucleotide sequence just internal to the 5′ cap has been found to be identical to 21 nucleotides adjacent to the poly(A) terminus at the 3′ end of the same molecule, approximately 10,000 nucleotides away. These reiterated terminal sequences appear to play a critical role in the circularization of proviral DNA, prior to its integration into the genome of the cell.

It appeared logical to try to inhibit the production of RSV by interfering with the circularization step, essential to the production of RSV, in infected chick embryo fibroblast (CEF) cells. A tridecamer deoxyribonucleotide, d(A-A-T-G-G-T-A-A-A-A-T-G-G), complementary to a 13-ribonucleotide segment of the reiterated terminal sequence was synthesized by John Hachmann of Collaborative Research, Inc., and was added to cultures of CEF cells at the time of their infection with RSV. Inhibition of production of RSV resulted, in the presence of either the tridecamer or a modified tridecamer in which the 3′- and 5′-terminal hydroxyl groups had been blocked as the isourea derivatives. In addition to the possible block of the circularization step, there are several other points at which interference with the life cycle of RSV might occur, as a result of "hybridization competition."

A detailed study of the inhibitory effect of the tridecamer on cell-free translation of the 35S RNA viral messenger is presented in a companion publication (3).

MATERIALS AND METHODS

RSV-ts68, originally from Kawai and Hanafusa (4), was obtained from Maureen Gammon, Gastrointestinal Laboratories, Massachusetts General Hospital. Chick embryo fibroblasts were obtained as primary explants from 10-day-old Spafas chicks, and were used at early passages. Tissue cultures were grown as monolayers, using 10% tryptose, 5% irradiated fetal calf serum, and Dulbecco's modified Eagle's medium, without antibiotics (5). Incubation was at 37° in 5% CO_2/95% air.

RNA-dependent DNA polymerase (reverse transcriptase) assays were performed with minor modifications of the procedures of Baltimore (6) and of Scolnick *et al.* (7). The assays were carried out on lysed 100,000 × *g* pellets from the 7500 × *g* supernatant of tissue culture medium. Focus and plaque assays were carried out by means of the caseinolytic technique of Balduzzi and Murphy (8). The tridecamer d(A-A-T-G-G-T-A-A-A-A-T-G-G) was synthesized by Collaborative Research, Inc., using modifications of the triester method of Narang and colleagues (9), and details will be published elsewhere (J. Hachmann, R. Knott, and A. Taunton-Rigby).

With the base and triester blocking groups still attached to the tridecamer, a portion of the material was subjected to blocking of the free terminal 5′- and 3′-OH moieties by John Hachmann, by addition of phenylisocyanate with formation of the isourea derivatives (10). Following removal of the triester and base blocking groups, the terminal 3′- and 5′-OH moieties remained blocked. This material we call "terminal blocked tridecamer." the remainder of the internally blocked tridecamer was also deblocked, to become free tridecamer.

The degree of purity of the free tridecamer was tested in the following way by Dennis Schwartz of these laboratories and by John Hachmann. ^{32}P from very high specific activity [γ-^{32}P]-ATP was added to the free terminal 5′-OH moiety of the tridecamer by polynucleotide kinase of a high degree of purity. It was found essential that the kinase be free of 3′→5′ exonuclease, in order to avoid a misleading underestimation of the degree of purity of the tridecamer.

Analysis of the labeled tridecamer by electrophoresis on cellulose acetate followed by homochromatography and radioautography (1) revealed only one major spot plus three small satellite spots. Our estimate is that the tridecamer is 90–95% pure. Two separate aliquots were deblocked and so treated, and both exhibited the same high degree of purity. In further experiments, sequence analysis was performed, employing partial enzymatic degradation of the labeled tridecamer with snake venom phosphodiesterase. Subsequent mobility shift analysis indicated that the sequence is as written: d(A-A-T-G-G-T-A-A-A-A-T-G-G).

The degree of purity of the 5′- and 3′-terminally blocked tridecamer is still not definitely determined, being estimated by a biological test, the decreased ability to act as a primer of

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Abbreviations: RSV, Rous sarcoma virus; CEF, chick embryo fibroblast; FFU, focus-forming unit; MOI, multiplicity of infection.

Table 1. Effect of blocked tridecamer on the appearance of reverse transcriptase activity in medium of CEF cells preexposed to RSV

Virus input, FFU	Blocked tridecamer, 0–48 hr	Flask	cpm of reverse transcriptase activity at 0–48 hr	48–96 hr	96–144 hr	144–192 hr	192–240 hr
Control	–	1	560	820	1,960	780	830
(no virus)			410	1,670	2,620	620	240
1×10^6	–	2	1,110	242,000			
MOI = 2			1,080	239,000			
	–	3	1,850	250,000			
			1,750	240,000			
1×10^5	–	4	410	124,000	85,000		
MOI = 0.2			280	122,000	81,000		
	–	5	280	72,000	78,000		
			90	68,000	84,000		
	+	6	0	14,000	75,000		
			120	12,600	46,000		
	+	7	2,300	8,000	26,000		
			0	9,700	25,000		
1×10^4	–	8		1,200	5,500		
MOI = 0.02					6,600		
	–	9		2,800	7,000		
				2,200	7,900		
	+	10		1,100	6,700		
				1,200	5,700		
	+	11		1,200	460		
				900	290		
1×10^3	–	12		0	0	16,000	117,000
MOI = 0.002				1,800	0	15,000	108,000
	–	13		1,200	0	7,300	139,000
				0	0	7,100	132,000
	+	14		900	0	2,100	630
				1,000	0	50	740
	+	15		0	0	630	2,400
				0	0	2,400	2,600

The virus–tissue culture procedure is essentially that of Hanafusa (5). Early passage CEF cells were trypsinized, planted in 25-cm^2 Falcon flasks, and incubated for 4 hr at 37° with 5% CO_2/95% air. Unattached cells and medium were removed, and 0.2 ml of virus suspension contained in 10% tryptose/2% fetal calf serum/DEAE-dextran at 5 μg/ml, in Dulbecco's modified Eagle's medium, was added to the attached CEF cells (approximately 5×10^5 cells per flask) for 90 min at 37°. Three milliliters of medium containing 10% tryptose and 5% fetal calf serum, in Dulbecco's modified Eagle's medium, was employed for the incubation medium thereafter. This medium included tridecamer or blocked tridecamer at 10 μg/ml where used, unless otherwise indicated. Terminally blocked tridecamer was used only for the first 48 hr of incubation; medium was changed every 48 hr thereafter.

The viral reverse transcriptase assay (6, 7) was carried out on lysed 100,000 × *g* pellets from 7500 × *g* supernatants of the tissue culture medium at the indicated times. Pellets from 3 ml of medium from each flask were lysed in 100 μl of lysing buffer (0.3% Triton X-100/0.05 M Tris·HCl, pH 8.1/0.05 M KCl/0.02 M dithiothreitol). The enzyme incubation was in a total volume of 100 μl, and 25-μl aliquots were taken for measuring radioactivity. Each 100-μl incubation mix contained 25 μl of enzyme solution in lysing buffer and had the following composition: 0.06 M Tris·HCl at pH 8.1, 0.06 M KCl, 0.006 M $MgCl_2$, 0.006 M dithiothreitol, 0.04 A_{260} unit of oligo(dT)·poly(A) (equimolar in nucleotides), 0.075% Triton X-100, and 0.2 mM [^{3}H]dTTP (3.0×10^6 cpm). Incubation was for 1 hr at 37° unless otherwise indicated.

Following incubation, 25-μl aliquots were taken, the tubes were chilled, and 0.6 ml of 0.1 M NaH_2PO_4 containing 100 μg of yeast carrier RNA was added, followed immediately by 0.3 ml of 30% trichloroacetic acid. The contents were mixed in a vortex mixer and allowed to stand 20 min in ice to precipitate the nucleic acids. The mixture was filtered onto Whatman GF/C 25-mm glass-fiber filters placed in 24-mm Hirsch funnels. The filters were washed eight times with 10 ml of cold 7% trichloroacetic acid containing 0.05 M sodium pyrophosphate and 0.05 M NaH_2PO_4, followed by a cold 95% ethanol wash. The filters were placed in scintillation vials and dried at 80° for at least 1 hr. The sample was solubilized by the addition of 75 μl of H_2O and 0.6 ml of NCS solubilizer (Amersham/Searle). The vials were capped and warmed in an oven at 40° for 1 hr. Ten milliliters of 0.3% 2,5-diphenyloxazole (PPO) and 0.03% 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP) in toluene was added and the samples were assayed using a Nuclear Chicago scintillation counter (having ^{3}H efficiency of about 40%). At least three 5-min counts were collected before the average number of cpm was calculated.

reverse transcriptase, and details are provided in the accompanying publication (3).

RESULTS

Table 1 illustrates the relation between virus input concentration and the development of reverse transcriptase activity in the high-speed pellets of the incubation medium from the virus-infected CEF cells in tissue culture. As the virus input concentration is decreased, the latent period before the rise in reverse transcriptase activity increases. In the 48- to 96-hr medium collection, the reverse transcriptase activity reaches a level around 340,000 cpm at a focus-forming unit (FFU) level of 1×10^6/ml of input virus. At a FFU input level of 1×10^3/ml and a multiplicity of infection (MOI) of 0.002 the reverse transcriptase level does not rise above background until 144–192 hr, and it reaches a level around 125,000 cpm only at 192–240 hr.

Table 2. Effect of tridecamer on the appearance of reverse transcriptase activity in medium of CEF cells preexposed to RSV

MOI	Tridecamer	Flask	cpm of reverse transcriptase activity at 0–48 hr 0°C	0–48 hr 37°C	0–48 hr 37°C	48–96 hr 0°C	48–96 hr 37°C	48–96 hr 37°C	96–144 hr 0°C	96–144 hr 37°C	96–144 hr 37°C
Control	–	1	180	170	130	270	310	280	250	270	270
(no virus)	–	2	130	110	120	200	240	320	240	260	260
1	–	3	160	2,040	2,030	550	21,300	20,800	1,290	82,200	81,200
	–	4	130	1,120	1,100	500	16,800	16,600	930	55,500	52,500
	+	5	140	1,200	1,200	320	7,180	6,670	—	—	—
	+	6	120	980	880	330	9,200	8,800	510	31,600	32,600
0.1	–	7	170	440	430	300	4,500	4,600	760	48,100	48,000
	–	8	170	190	200	300	2,300	2,300	720	40,400	39,000
	+ (20 μg/ml)	9	120	250	240	270	1,800	1,800	360	11,900	11,400
	+ (80 μg/ml)	10	160	180	220	250	1,070	1,120	530	30,200	32,200

Control exp.	Material assayed	cpm of reverse transcriptase activity 0°C	37°C	37°C
1	Lysis buffer	130	85	100
	AMV reverse transcriptase	5,700	344,000	340,000
2	Lysis buffer	180	190	190
	AMV reverse transcriptase	6,300	407,000	433,000

The methods were as described for Table 1, except that tridecamer was added with each medium change, up to termination of the experiment at 144 hr. For description of flask 10, see text. For control experiments, purified reverse transcriptase from avian myeloblastosis virus (AMV) was used. The counter background was 27 cpm.

The CEF flasks treated with terminally blocked tridecamer, only for the first 48 hr after addition of virus and incubation, show a marked decrease in virus output into the incubation medium, as measured by reverse transcriptase activity in the high-speed pellets. Flasks 14 and 15 (Table 1) show an average inhibition of virus production of 99% at 192–240 hr. Flasks 6 and 7, with a MOI = 0.2, show an approximate 90% inhibition of virus output at the 48–96-hr collection time period. It may be noted that these same flasks, 6 and 7, show an escape from the inhibition imposed by the presence of blocked tridecamer in the medium for the first 48 hr, during the time period 96–144 hr. This escape effect we have also observed in the case of the unblocked tridecamer, following its omission from the incubation medium at high MOI of added virus.

Less dramatic but similar inhibitory effects on virus production are shown in Table 2. Here the unblocked tridecamer is used, at a concentration (10 μg/ml; ~2 μM) similar to that of the terminally blocked tridecamer in Table 1. A high MOI, that is, 1:1, is used in flasks 3–6, and 1:10 in flasks 7–10.

We have labeled the 5′-OH terminus of the tridecamer with ^{32}P, using polynucleotide kinase and [γ-^{32}P]ATP, as described in *Materials and Methods*, and have looked for evidence of cell penetration and association of the labeled tridecamer with 35S RSV RNA with virus-infected CEF cell cultures. The terminal ^{32}P label, however, is very rapidly removed from the tridecamer by hydrolysis due to phosphomonoesterase in the incubation medium. We have found ^{32}P label that travels in a sucrose gradient (11) in the region of 32S RNA, when total RNA is extracted from virus-infected CEF cells incubated with 5′-^{32}P-terminally labeled tridecamer. It remains uncertain whether this label is due to reincorporation of $^{32}P_i$ into RNA following 5′-terminal phosphomonoester hydrolysis, or whether this represents hybridization of labeled tridecamer with viral mRNA within the virus-infected cells. That the tridecamer does hybridize *in vitro* with RSV 35S RNA is shown by its ability to act as an excellent primer for the reverse transcriptase, using RSV 35S RNA [as described in the accompanying publication (3)].

The terminally blocked tridecamer has the advantage over the unblocked tridecamer of being presumably less susceptible to exonucleolytic enzymes present in the serum-containing incubation medium and within the cells. It may therefore have a longer half-life within the CEF cells.

The inhibitory effect of the blocked tridecamer and the unblocked tridecamer on virus production is paralleled by visual observation, through the phase microscope, of decreased cell transformation in CEF cultures where these tridecamers have been used. Plaque and focus-forming assays, using the agar-caseinolytic technique (8), also show inhibition where supernatant media from the tridecamer-treated cultures, taken at appropriate time intervals, are brought in contact with CEF cells, overlaid with casein–agar medium, and compared with sister cultures that have had no tridecamer added (i.e., flasks 14 and 15 versus flasks 12 and 13, Table 1).

In one instance (Table 2, flask 10), 80 μg of unblocked tridecamer per ml of culture medium was used for 4 days in a CEF culture with an initial MOI = 0.1. The inhibition was nearly twice that found in a sister culture exposed to 20 μg tridecamer per ml of culture medium for the same period of time. Following the second change of medium, at 96 hr, no more tridecamer was added to the culture exposed to tridecamer at 80 μg/ml. The reverse transcriptase activity rose rapidly during the subsequent 48 hr. In this and other experiments, therefore, the inhibition induced at relatively high MOI values is reversible (cf. also flasks 6 and 7, Table 1).

We have at this early stage in these studies seen no overt evidence of toxicity of the tridecamer to normal CEF cells in the concentrations usually employed (10 μg/ml). In a typical experiment, employing 25-cm^2 Falcon flasks for monolayer cultures, approximately 5×10^5 cells are used for initial infection. At the end of 6 days, around 5×10^6 cells are present in all cell cultures: i.e., control, uninfected cultures, cultures

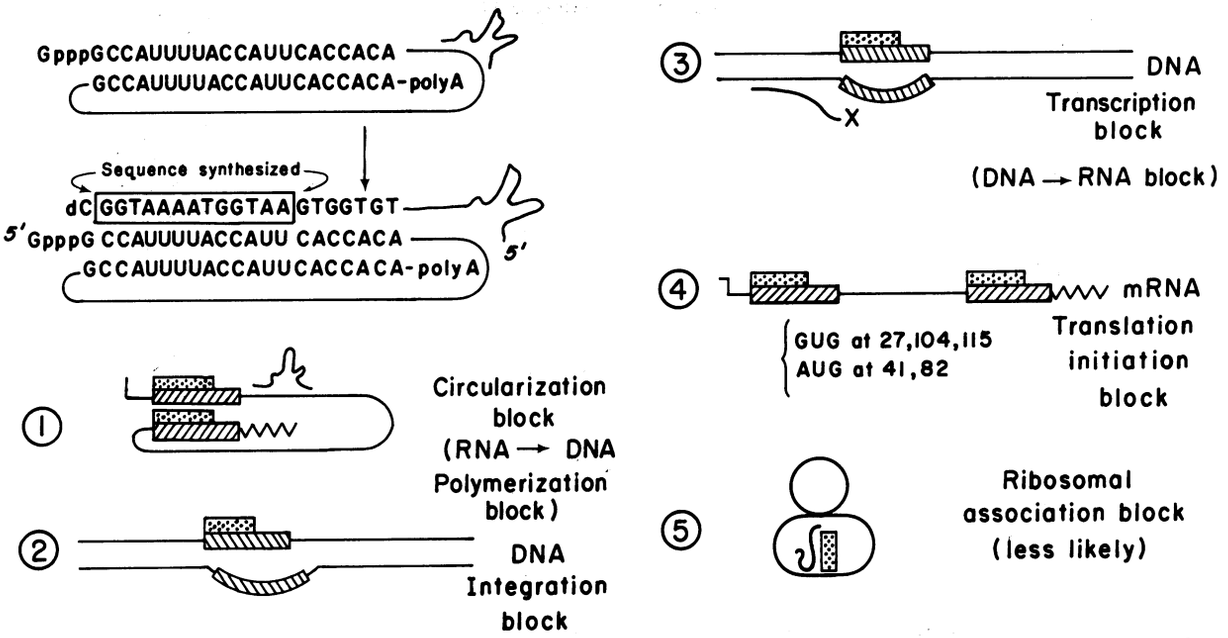

FIG. 1. Possible sites of action of tridecamer. ▨, Tridecamer, hydrogen bonded to single-stranded RNA or DNA. ▨, Reiterated terminal sequences in RNA, which are a part of the RNA molecule represented as a solid line. ▨, Presumed integration sites in DNA; the single strands of DNA are represented as single lines joined to these integration sites. Primer tRNA is represented as a modified clover leaf.

infected at time 0 with RSV, cultures infected with RSV with tridecamer added, and cultures with tridecamer added without RSV. It seems unlikely therefore that the tridecamer acts via a general inhibition of mitosis.

We have also added tridecamer to CEF cultures already producing RSV at a rapid rate, with a preponderance of cells visibly transformed. Four days after virus addition, 4 out of 10 flasks were treated with tridecamer (10 μg/ml of incubation medium). Twenty-four hours later the reverse transcriptase assay indicated an inhibition of virus production in the tridecamer-treated flasks. By the standard t test the difference was significant, at a value of $P < 0.01$.

DISCUSSION

The greatest inhibition of virus production in the presence of a constant concentration of tridecamer has been observed where the multiplicity of infection is least (see Table 1). Here the latent period for transformation and virus production is longest. It would appear logical to expect a viral competitor to have the greatest chance of success under such circumstances.

There are several points in the life cycle of the RSV in the CEF cell in tissue culture at which a hybridization competitor oligodeoxynucleotide might act, as illustrated in Fig. 1. An attractive possibility is a circularization block (No. 1, Fig. 1), because it appears from a variety of evidence (12–17) that terminal redundancy and a circular DNA proviral intermediate are essential for integration of viral information into the genome of the CEF cell. That translation initiation blockade (No. 5, Fig. 1) is an *in vitro* reality is documented in the accompanying paper (3). A possible inhibitory site not depicted in Fig. 1 is at a splicing step involved in formation of mRNA.

Employment of an oligodeoxynucleotide hybridization competitor to inhibit oncogenic viral integration, multiplication, and cell transformation offers a novel approach to the problem of oncogenesis. Its possible future general chemotherapeutic potentiality depends first on sequencing RNAs (or DNAs) from oncogenic viruses to which particular animal species are susceptible, and then synthesizing deoxyribo- or ribo-oligonucleotides that are of sufficient length to hybridize well with a segment of viral RNA or DNA, and are sufficiently specific for the virus in question (nononcogenic as well as oncogenic) to avoid hybridizing well with normal cellular nucleic acids. For example, if the primary sequence of a unique small segment of a negative strand RNA virus such as influenza, measles, or rabies were known, it would be possible to synthesize and test its oligonucleotide complement as a possible virus inhibitor. (Our colleague Jesse F. Scott suggests the term "hybridon" to characterize an oligonucleotide of specified sequence that acts in a metabolic sense by competitive hybridization.)

Note Added in Proof. The following related publications have come to our attention: Tennant *et al.* (18) have found an inhibitory effect of poly(A) and poly(*O*-methyl-A) on murine oncornavirus production in tissue culture, and inhibition largely due to impairment of penetration of virus into the host cell. Miller *et al.* (19) report that the trinucleotide complement to the -C-C-A terminus of tRNA inhibits protein synthesis by 40% when added to mammalian cells in tissue culture.

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