5'-Terminal Nucleotide Sequences of Mammalian Type C Helper Viruses Are Conserved in the Genomes of Replication-Defective Mammalian Transforming Viruses

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The RNAs of replication-defective murine and primate type C transforming viruses were analyzed for the presence of nucleotide sequences homologous to the genomes of their respective helper type C viruses by using DNAs complementary (cDNA) to either the 5'-terminal (cDNA^{5'}) or total (cDNA_{total}) nucleotide sequences of the helper virus RNA. The defective viruses examined have previously been shown to vary in their ability to express helper viral *gag* gene proteins. With cDNA_{total} as a probe, these transforming viruses were shown to vary in their representation of helper virus sequences (15 to 60% hybridization of cDNA_{total}). In striking contrast, 5'-terminal-specific sequences of the helper virus were conserved in the RNAs of every transforming virus tested (>80% hybridization of cDNA^{5'}). These findings suggest a critical role for these sequences in the life cycle of the defective transforming virus.

Type C retroviruses, which induce solid or hematopoietic tumors and cause transformation of fibroblasts in vitro, have been isolated from a number of mammalian species. Such transforming viruses are defective in that they require a type C helper virus that supplies functions necessary for replication (1, 2, 6, 21, 23, 26, 34). Accumulating evidence indicates that the genome of the transforming virus is composed of unique nucleotide sequences in addition to some sequences homologous to those of a type C helper virus (4, 8, 15, 17, 22, 27, 33, 36). The extent of representation and expression of helper viral sequences varies among different transforming virus isolates (5, 15, 29, 32, 33). The helper and transforming virus-specific nucleotide sequences appear to be covalently linked (15, 22), but their exact arrangement is as yet unresolved.

Recently, a potentially useful approach to analyze the genomes of mammalian transforming viruses has come from the demonstration that complementary DNA (cDNA) probes specific for the 5' region of helper viral RNA can be synthesized in vitro under defined conditions (12, 18, 19). In the present report, a large number of mammalian transforming viruses have been analyzed for their representation of helper viral sequences by using probes generated from total or 5'-terminal regions of the helper virus RNA. The results demonstrate that a general property

of mammalian type C transforming viruses is their conservation of 5'-terminal helper virus genetic sequences. The implications of these findings with regard to the structure and life cycle of replication-defective transforming viruses are discussed.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Dulbecco's modification of Eagle medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.). Cell lines included those of mouse (NIH/3T3), rat (NRK), and human (A204 and HOS) (5) origin. The derivations of cell lines nonproductively transformed by different replication-defective viruses are cited in the text. Type C RNA helper viruses, obtained through the auspices of the Office of Resources and Logistics, National Cancer Institute, included Moloney and Kirsten strains of mouse leukemia virus (MuLV), propagated in NIH/3T3 or NRK cells; woolly monkey helper virus, grown in NRK or A204 cells; and RD114 virus, grown in A204 cells.

Preparation of viral cDNA's. Single-stranded viral cDNA's complementary to the RNAs of Moloney MuLV or woolly monkey helper virus were synthesized in reactions that included: 20 mM Tris-hydrochloride, pH 7.8; 6 mM KCl; 1 mM dithiothreitol; 6 mM MgCl₂; 0.5 mM (each) dATP, dCTP, and dGTP and 0.05 mM [³H]TTP (54.4 Ci/mmol; New England Nuclear Corp., Boston, Mass.); 50 μg of actinomycin D per ml; 0.8 mg of DNase I-digested calf thymus DNA (38) per ml; 0.014% Triton X-100; and 100 to 500 μg of virus per ml. The ³H-labeled cDNA's were purified as

previously described (32). The average size of such cDNA's, determined by alkaline sucrose gradient centrifugation, was 160 nucleotides.

DNA complementary to the 5' end (cDNA^{5'}) of the Moloney MuLV or woolly monkey helper viral genome was synthesized in a reaction that contained 0.02 M Tris-hydrochloride, pH 8.3; 1 mM dithiothreitol; 6 mM MgCl₂; 1 mM (each) dATP, dGTP, and dTTP and 4 μ M [α -³²P]dCTP (specific activity ~ 200 Ci/mmol; New England Nuclear); 100 μ g of actinomycin D per ml, 0.02% Nonidet P-40, 50 U of purified avian myeloblastosis virus reverse transcriptase (University Laboratories, Highland Park, N.J.); and 100 μ g of 70S viral RNA (prepared as previously described [19]) per ml. Discrete size classes of cDNA^{5'}s were isolated by polyacrylamide gel electrophoresis under denaturing conditions (20).

RNA purification. Viral 70S RNA and cellular RNA were purified exactly as described in reference 16.

Molecular hybridization. cDNA-RNA hybridization was performed in reaction mixtures containing 0.6 N NaCl, 0.01 M Tris (pH 7.5), 0.1 mM EDTA, 500 to 1,000 cpm of cDNA, and varying amounts of RNA at 62°C for 18 to 48 h. Hybrid formation was determined by using S1 nuclease (8). Data are expressed as a function of C_t (RNA concentration in moles of nucleotide per liter $\times$ time in seconds [9]). C_t values have been corrected to a monovalent cation concentration of 0.18 M (10).

RESULTS

Characterization of Moloney MuLV cDNA probes. To investigate the representation of murine helper virus sequences in murine transforming viruses, three sets of cDNA probes were prepared from a prototype MuLV, Moloney MuLV. DNA complementary to the Moloney MuLV RNA was synthesized in reactions containing a large excess of oligodeoxynucleotides generated from calf thymus DNA, which served to prime DNA synthesis along the entire viral genome (38). The cDNA generated in this way protected more than 80% of ³²P_i-labeled Moloney MuLV 70S RNA from digestion by RNase at a cDNA:RNA ratio of 2 (data not shown), indicating that this cDNA was a reasonably uniform transcript of the viral RNA. This probe was designated cDNA_{total}.

DNA fragments complementary to the 5' end

of the Moloney MuLV genome were synthesized under conditions in which the initiation of DNA synthesis occurs at the tRNA primer, 135 nucleotides from the 5' end of the genome (19; J. M. Coffin, A. M. Maxam, and W. A. Haseltine, Cell, in press). The initial DNA product synthesized represents transcription of the 5'-terminal 135 nucleotides. When the concentration of one of the deoxynucleotide triphosphates in the reaction mixture is limiting, termination products are observed that give rise to a series of cDNA's shorter than 135 nucleotides. Such cDNA's, designated cDNA_n^{5'}, where *n* is the length in number of nucleotides, were purified on polyacrylamide gels and used as probes. To determine the purity of these DNA fragments, a fraction of each was depurinated, and the oligopyrimidines were analyzed by electrophoresis followed by homochromatography (19). By comparison of the oligopyrimidine composition of these DNA segments with the known sequence of Moloney MuLV cDNA₁₃₅^{5'} (A. M. Maxam, T. C. Hageman, and W. A. Haseltine, manuscript in preparation) both the origin and the purity (>90%) of these segments were confirmed.

The Moloney MuLV genome has been shown to contain a terminally redundant sequence approximately 55 nucleotides long (Coffin et al., in press). Thus, cDNA₁₃₅^{5'} contains a sequence complementary to both the 3' and the 5' termini of the genome. cDNA products less than or equal to about 80 nucleotides long are copied from a sequence located between the tRNA binding site and the terminal redundancy. Analysis of the oligonucleotides derived by RNase T₁ digestion of short, polyadenylic acid-containing viral RNA (200 to 300 nucleotides long) shows that this sequence is not present at the extreme 3'-terminal end of the genome (Coffin et al., in press). Figure 1 provides a schematic illustration of the origins of the Moloney MuLV cDNA₁₃₅^{5'} and cDNA₄₀^{5'} used in the present studies.

The kinetics of hybridization of the Moloney MuLV cDNA probes (cDNA_{total}, cDNA₁₃₅^{5'}, and cDNA₄₀^{5'}) with Moloney MuLV 70S RNA were examined. Greater than 95% of each of the Moloney MuLV cDNA's was hybridized, although the C_t_{1/2} value for cDNA₄₀^{5'} was significantly

FIG. 1. Schematic diagram of the sites of synthesis of Moloney MuLV cDNA^{5'}s. The diagram is based on data presented in reference 19 and in Coffin et al., in press.

higher than those of the other probes (Table 1). These results are most readily explained by the known dependence of hybridization reaction rate on DNA fragment size (41). Thus, to ensure saturation hybridization with cDNA₄₀^{5'}, reactions with this probe were carried out to a C_t value 10-fold in excess of that required to achieve maximum hybridization with cDNA_{total} and cDNA₁₃₅^{5'}. The *T_m* values indicated the formation of well-matched hybrids between Moloney MuLV RNA and each of the cDNA probes (Table 1); the *T_m* values of hybrids with cDNA₄₀^{5'} were appreciably lower ($\Delta T_m = 3$ to 5°C), as might be expected for smaller hybrids (25).

To ascertain whether the Moloney MuLV cDNA probes could be used to detect murine helper viral sequences other than those of Moloney MuLV, hybridization studies were performed with another murine helper virus, Kirsten MuLV (Table 1). Nucleotide sequence homology with Kirsten MuLV RNA was demonstrated for each cDNA. Moloney MuLV cDNA₄₀^{5'} exhibited significantly less homology to Kirsten MuLV RNA than did cDNA₁₃₅^{5'}, suggesting greater sequence divergence of the unique portion of the 5'-terminal end of the Kirsten MuLV genome. The lower thermal stabilities of hybrids formed between each cDNA and Kirsten MuLV RNA as compared with those formed with Moloney MuLV RNA suggested that the lower extents of hybridization observed with Kirsten MuLV RNA resulted from random base pair mismatching (Table 1).

Analysis of the RNAs of murine transforming viruses with Moloney MuLV cDNA^{5'}s. To test for the presence of specific helper viral sequences in murine transforming viruses, studies were performed with RNAs of cells nonproductively transformed by a variety of such viruses. S⁺L⁻ Moloney murine sarcoma virus (MSV) and Abelson leukemia virus each

code for Moloney MuLV type-specific *gag* gene products (5, 29), strongly implying that the helper virus sequences associated with their genomes were contributed by Moloney MuLV. The nonproducer strain of Moloney MSV and Harvey MSV do not code for any detectable helper viral proteins (5, 29). However, these viruses have also been reported to contain Moloney MuLV nucleotide sequences, as determined by molecular hybridization (29). RNAs of cells nonproductively transformed by each of these viruses hybridized Moloney MuLV cDNA_{total} to different extents, ranging from 15% for Harvey MSV to 60% for S⁺L⁻ Moloney MSV (Table 2). In striking contrast, from 74 to 88% of both cDNA₁₃₅^{5'} and cDNA₄₀^{5'} were hybridized by these same RNAs, demonstrating the conservation of helper virus 5'-specific sequences. Representative hybridization kinetics of each cDNA with the RNAs of two transforming viruses are presented in Fig. 2.

Table 2 indicates analogous findings for transforming viruses isolated in association with murine helper viruses other than Moloney MuLV. RNA of NRK cells nonproductively transformed by either Kirsten MSV or BALB MSV hybridized 7 and 27% of cDNA_{total}, respectively. Since these genomes contain helper viral sequences of ecotropic viruses only partially related to Moloney MuLV, the representation of helper viral sequences in these transforming viruses is likely to be underestimated by use of Moloney MuLV cDNA_{total} as a probe (Table 1). The extent of hybridization achieved with cDNA₄₀^{5'} (40%) reflects very similar results obtained with the RNA of Kirsten MuLV (Table 1). The presence of 5'-terminal helper virus sequences in these sarcoma viruses was demonstrated by findings of greater than 70% annealing of these RNAs to Moloney MuLV cDNA₁₃₅^{5'}.

The specificity of the hybridization reactions was indicated by the observation that RNAs isolated from either rat or human cells transformed by the same virus yielded the same hybridization values. None of the probes reacted with RNA of rat cells transformed by Rous sarcoma virus or with uninfected rat or human cells. In addition, when Kirsten MSV was rescued by a helper virus, RD114, 50-60S RNA of the pseudotype virus hybridized with each probe to the same extent as that observed with the RNA of Kirsten MSV nonproducer cells. No hybridization was observed between the Moloney MuLV cDNA probes and RD114 RNA alone.

5'-Terminal helper virus sequences in clonal variants of a primate transforming virus. Previous studies have demonstrated that clonal variants of a naturally occurring woolly

TABLE 1. Hybridization of Moloney-MuLV cDNAs with murine type C helper viral RNAs^a

cDNA	70S RNA source					
	Moloney MuLV			Kirsten MuLV		
	Maximum hybridization (%)	C _t _{1/2} ^b (×10 ⁻²)	<i>T_m</i>	Maximum hybridization (%)	C _t _{1/2} ^b (×10 ⁻¹)	<i>T_m</i>
cDNA _{total}	95	3.8	87	46	1.2	79
cDNA ₁₃₅ ^{5'}	98	2	88	79	1.0	82
cDNA ₄₀ ^{5'}	98	19.5	82	43	6.0	75

^a Hybridization reactions were performed as described in the text and carried out to a C_t value of 5.0.

^b C_t_{1/2} = C_t value at which one-half maximum hybridization was achieved.

TABLE 2. Presence of helper virus 5' terminal nucleotide sequences in RNAs of murine transforming viruses

RNA source	Cell line designation ^a	Mouse helper virus proteins expressed	Maximum hybridization with Moloney MuLV (%) ^b		
			cDNA _{total}	cDNA ₁₃₅ ^{5'}	cDNA ₄₀ ^{5'}
Cells nonproductively trans- formed by:					
S ⁺ L ⁻ Moloney MSV	S ⁺ L ⁻ NRK (rat)	p15, p12, p30	60	88	82
S ⁺ L ⁻ Moloney MSV	HuAC1 ₁ (human)	p15, p12, p30	57	87	81
Abelson leukemia virus	AB-NRK (rat)	p15, p12	59	86	84
Nonproducer Moloney MSV	M-NRK C1 1-2 (rat)	None	26	88	84
Harvey MSV	H-NRK (rat)	None	15	84	74
BALB MSV	MAI-NRK C1 4 (rat)	p15, p12	27	77	38
Kirsten MSV	KNRK C1 32 (rat)	None	7	73	41
Kirsten MSV	KHOS (human)	None	8	75	40
Schmidt-Ruppin avian sarcoma virus	SR-NRK C1 E (rat)	None	<5	<5	<5
Uninfected cells					
Rat	NRK	None	<5	<5	<5
Human	HOS	None	<5	<5	<5
Virions					
Kirsten MSV (RD114)	HOS		7	75	42
RD114	HOS		<5	<5	<5

^a Reference 5 cites studies describing the derivation and immunological analysis of each nonproducer cell line tested.

^b Reactions were carried out to a C_{t} value of 5×10^3 . Each reaction contained 500 to 1,000 cpm of cDNA. The values shown represent the actual extents of hybridization observed.

FIG. 2. Hybridization of Moloney MuLV cDNA's with RNA isolated from MSV-transformed nonproducer cells which differ in degree of expression of Moloney MuLV gag proteins. cDNA_{total} (A), cDNA_{5'135} (B), and cDNA_{5'40} (C) were each incubated with varying amounts of total cellular RNAs isolated from NRK cells transformed by the nonproducer strain of Moloney MSV (helper virus protein not expressed) (□) and from the S⁺L⁻ strain of Moloney MSV (Moloney MuLV p12, p15, and p30 expressed) (○). As controls, the probes were also incubated with total RNA purified from NRK cells chronically infected with Moloney MuLV (●) and from uninfected NRK cells (△). Hybridization reactions and S1 nuclease assays were carried out as described in the text.

monkey sarcoma virus (WSV) code for different numbers of woolly monkey helper virus-specific gag gene products (3, 5). To investigate the presence of 5' helper viral sequences in these WSV variants, woolly monkey helper virus cDNA_{total} or cDNA_{5'} probes were synthesized as described in Materials and Methods. For each

of the nonproducer transformed clones, the final extent of hybridization of the cellular RNA to woolly monkey viral cDNA_{total} was considerably less than it was for cells productively infected with the woolly monkey helper virus (Fig. 3A). The differences in extent of maximum hybridization are most likely due to deletions of portions

FIG. 3. Hybridization of woolly monkey helper virus cDNA's isolated from NRK cells nonproductively transformed by clonal variants of WSV. RNAs of WSV nonproducer cells expressing different woolly monkey helper virus gag proteins (5, 8) were hybridized with cDNA_{total} (A) and cDNA_{5'35'} (B). The WSV clones examined were the following: clone 9-3, negative for helper virus gag proteins, p12, p30, and p10 (●); clone 2-8, helper virus p12 expressed (○); and clone 11-2-3, helper virus p12, p10, and p30 expressed (△). RNAs from NRK cells chronically infected with woolly monkey helper virus (□) or nonproductively transformed by Schmidt-Ruppin avian sarcoma virus (▽) and uninfected NRK cells (■) were also tested for their ability to hybridize with cDNA_{total} and cDNA_{5'35'}. The hybridization values have been normalized to the maximum percent hybridization value for each cDNA with woolly monkey helper virus RNA (cDNA_{total} = 100%; cDNA_{5'35'} = 92%).

of the helper virus genome in the defective sarcoma genomes. This conclusion is supported by the findings that the genomic RNAs of the WSV variants are smaller than that of the woolly monkey helper virus (32, 35). Markedly different hybridization values were obtained with woolly monkey helper viral cDNA_{5'35'} (Fig. 3B). Each of the same WSV RNAs annealed cDNA_{5'35'} greater than 80%, a value comparable to the extent of hybridization of this cDNA to the cellular RNA of productively infected cells. Similar results were obtained with cDNA_{80'} and cDNA_{50'} (data not shown). As controls, RNAs of uninfected NRK cells and RSV-transformed NRK cells failed to react with the same cDNA's. Thus, these results demonstrate the conservation of 5'-terminal sequences relative to total helper viral nucleotide sequences in variants of a primate-derived sarcoma virus.

DISCUSSION

The ability to synthesize cDNA_{5'} of the type C helper viral RNA has provided a means to determine whether a specific region of the helper virus is contained within the genomes of mammalian transforming viruses. In the present report, cDNA's were generated from the 5' regions of murine (Moloney MuLV) and primate (woolly monkey) helper viruses. A series of cell lines nonproductively transformed by either murine or primate transforming viruses were examined with appropriate cDNA's as well as with

cDNA_{total} of the helper viral genome. The extent of representation of sequences homologous to the helper viral cDNA_{total} varied greatly among the transforming viruses analyzed. However, in all cases, extensive conservation of the helper 5' terminus was demonstrated, as defined by cDNA_{5'35'}. By use of shorter cDNA fragments, representing the 5' but not the extreme 3' terminus of the Moloney MuLV RNA, it was possible to demonstrate that transforming viruses, containing helper viral sequences of Moloney MuLV, conserved those 5'-specific nucleotides. Recent studies have revealed that helper viral information contained within the Moloney MSV genome is located at a position approximately 2,700 nucleotides from the 3' terminus and extends at least 3,000 nucleotides toward the 5' terminus (14, 22). It has also been reported that the Moloney MSV genome is terminally redundant (11). These results suggest that the helper viral 5'-terminal sequences studied here are localized to the 5' terminus of the defective transforming virus genome.

Previous studies have indicated that certain mammalian transforming viruses express *gag* gene but not other proteins specific to the helper virus initially isolated in association with them (3, 5, 29). The *gag* gene of the helper virus is thought to be localized to the 5' terminus of the viral genome (40). If, as the present studies indicate, mammalian transforming viruses invariably contain 5'-terminal helper viral nucleotide sequences, close linkage with helper viral *gag*

gene sequences might be expected as well. One possible explanation for the lack of expression of *gag* gene products by certain transforming viruses could be deletion of such sequences, as has been reported for different variants of Moloney MSV (29). Recent studies have suggested a different mechanism for the varying expression of *gag* gene products by stable variants of WSV (32). Molecular hybridization studies have indicated the preservation of *gag* gene nucleotide sequences of the helper virus by these variants, whether or not they express proteins encoded within the *gag* region of the helper virus genome. Such findings suggest that the lack of expression of *gag* gene products by certain WSV variants is due instead to point mutations, leading to premature termination of protein synthesis or the generation of nonsense polypeptides. This latter mechanism is also consistent with the present findings that the same WSV variants were found to possess 5'-terminal helper viral sequences.

The conservation of helper viral 5'-terminal sequences in replication-defective transforming viruses suggests an obligatory role of these sequences in the life cycle of the transforming virus. Recent studies have indicated that reverse transcription and the synthesis of *gag* and *pol* gene products are initiated at the 5' terminus of the helper viral genome (12, 13, 18, 19, 24, 30, 31, 37, 39). Moreover, electron microscopy of the helper RNA has indicated that RNA subunits interact at their 5' ends (7). Finally, the 5' regions of the viral RNA have recently been reported to provide leader sequences that may be necessary for the synthesis of active mRNA (28). Thus, evolutionary pressures exerted on transforming viruses that are obliged to replicate in association with a type C helper virus are likely to be highly selective for maintenance of critical functions associated with the 5'-terminal nucleotide sequences of the helper viral genome.

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