Tissue-Specific Expression in Transgenic Mice of a Fused Gene Containing RSV Terminal Sequences

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Transgenic mice were generated with pRSV-CAT, a chimeric gene construct containing the long terminal repeat of Rous sarcoma virus (RSV) linked to the bacterial gene encoding chloramphenicol acetyltransferase (CAT). CAT expression, detected in adult animals of five independent strains, was preferentially directed to organs rich in tendon, bone, and muscle. This pattern reflects the disease specificity of the intact virus and suggests that the tissue tropism of RSV is determined at least in part by the presence of endogenous tissue-specific factors that can promote expression of genetic information linked to the long terminal repeat. In two of the mouse strains, insertion of the pRSV-CAT DNA resulted in developmental abnormalities. One of these strains was characterized by a dominant trait of embryonic lethality, the other by a recessive trait of fused toes in all four feet.

THE VARIOUS ROUS SARCOMA VIRUS (RSV) strains originate from cell-free filtrates of a chicken spindle-cell sarcoma (*1*). In birds, these viruses have been implicated in the etiology of a variety of tumors that arise from mesenchymal tissue composed of cells such as fibroblasts, muscle cells, chondrocytes, and the like (*2*). Similar tumors can also be induced in rodents by infection with RSV (*3*). It appears, therefore, that in a broad range of vertebrate organisms, RSV can cause abnormal growth in cells or tissues of mesodermal origin. The affinity of RSV for specific tissues is only one example of the well-known phenomenon of viral tissue tropism. The molecular basis for viral tissue tropism has been linked to viral regulatory elements that respond to tissue-specific stimuli. In RSV as well as in many other retroviruses, regulatory elements have been located within or close to the long terminal repeat (LTR) of the proviral genome (*4*).

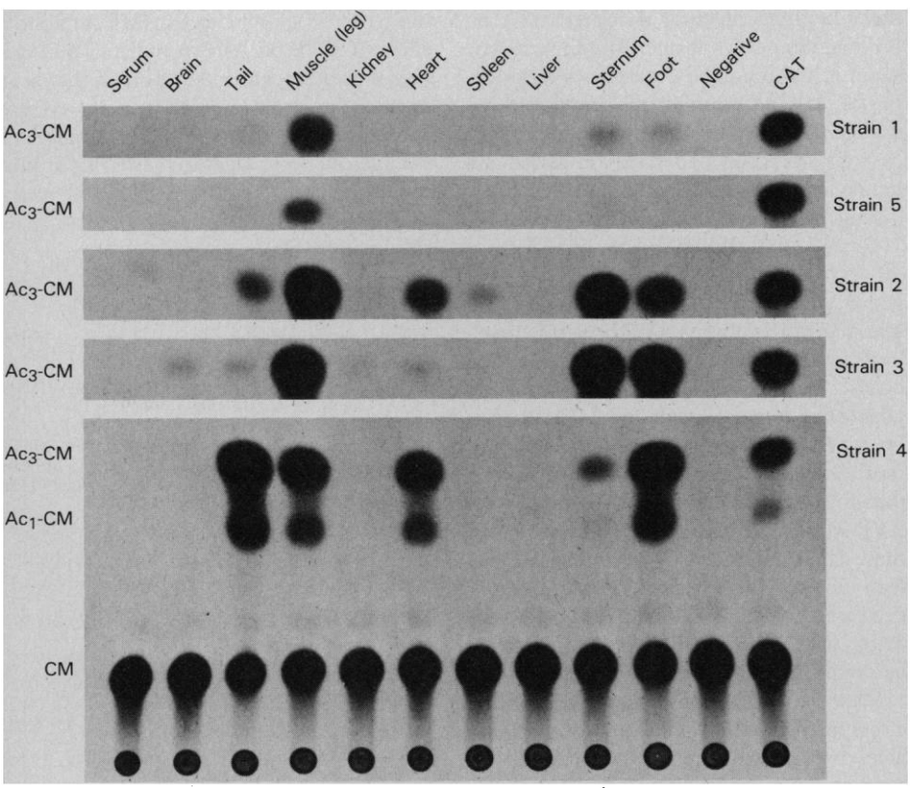

Fig. 1. CAT enzyme assays. Assays (*7, 16*) were performed on various tissues of representative adult F_1 mice of the five strains that show CAT activity. Tissues were homogenized in 250 m*M* tris-HCl (*p*H 7.8) by means of a Tekmar Tissumizer. After being heated to 65°C for 6 minutes to inactivate endogenous inhibitory factors (*18*), the homogenates were centrifuged in a microfuge for 10 minutes at 4°C. The supernatants were assayed for CAT activity by incubation for 90 minutes at 37°C with 0.2 μCi of [^{14}C]chloramphenicol (40 mCi/mmol, New England Nuclear) and 0.4 m*M* acetyl coenzyme A in 250 m*M* tris-HCl (*p*H 7.8). Assays were stopped by extraction with ethyl acetate and were analyzed by ascending thin-layer chromatography in chloroform:methanol (95:5) and subsequent autoradiography. Each assay contained 2 μg of tissue protein. Protein concentrations were determined with a dye reagent from Bio-Rad. The unacetylated (CM) and monoacetylated (Ac_1-CM, Ac_3-CM) forms of chloramphenicol are shown for strain 4. For strains 1, 5, 2, and 3, the 3-acetyl chloramphenicol spot only is shown. CAT, 5 μU of bacterial CAT enzyme.

In an effort to analyze LTR-mediated tissue tropism in a direct and comprehensive way, we decided to generate conditions that would allow us to examine every tissue in the organism for its potential to activate LTR-encoded control elements. Transgenic mice constitute an ideal system for this type of experiment. They are generated by microinjection of the gene of choice into newly fertilized embryos (*5*). In a fraction of the injected embryos, the DNA becomes stably integrated and is transmitted to subsequent generations. The new genetic information is present in every cell, so that all tissues at any developmental stage can be analyzed for expression of the inserted gene. Several recent studies have uncovered tissue specificities exerted by viral gene controlling sequences in transgenic mice (*6*). The gene construct that we selected for insertion in the mouse genome, pRSV-CAT, consists of RSV LTR sequences fused to the 5′ end of the coding sequences for the bacterial enzyme chloramphenicol acetyltransferase (CAT) (*7*). The RSV sequences included in pRSV-CAT contain transcriptional control elements with cell specificity (*8*), and the plasmid is actively expressed in mammalian cells (*7*). CAT is easily assayed, and there is no comparable activity in normal mice. A CAT signal in any tissue of a transgenic mouse would indicate the presence of endogenous factors conducive for expression of pRSV-CAT from its integrated state. We now report the expression of the CAT gene in specific tissues and organs of five transgenic strains carrying pRSV-CAT sequences.

Our study began with an examination of the presence and expression of the transferred pRSV-CAT gene in F_0 mice; these were animals that had been injected at the zygote stage. The numbers of integrated gene copies ranged from 1 to almost 200 (Table 1). Southern analysis revealed the presence of multimeric, head-to-tail arrangements of the gene in the strains carrying multiple copies. This type of arrangement at the site of integration has been reported in

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several studies (*5*). Most of our transgenic F_0 mice transmitted the pRSV-CAT sequences to approximately 50 percent of their offspring, indicating a single site of integration in the genome. Strain 4 had a low transmission rate (14 percent), suggesting that the F_0 mouse was a mosaic. The high transmission rate (91 percent) of the F_0 mouse of strain 5 is due to the presence of more than one site of integration. This mouse was also found to have an unusually small average litter size (3.8 newborns per litter).

Upon breeding [(wild type) $F_0 \times$ wt, $F_1 \times F_1$], transgenic mice of strains 1 to 4 consistently expressed CAT in their tails. Some, but not all, of the transgenic offspring of strain 5 were also CAT-positive, although no CAT activity was detected in the F_0 mouse. Organ screens for CAT activity were performed to determine the tissue specificity of pRSV-CAT expression (Fig. 1 and Table 2). The highest levels of CAT activity were found in leg muscle, abdominal muscle, sternum, foot, tail, and heart. CAT activity in high-expressor strains was approximately 100 times greater than that in low-expressor strains. No correlation between gene copy number and level of expression was apparent. No CAT activity was found in any organs of the mice of strains 6 to 9. The absence of CAT activity in strains 7 and 8 may have been due to integration of incomplete CAT coding sequences, since these strains carry less than one copy of RSV-CAT DNA per haploid genome. The lack of expression in strains 6 and 9 and differences in levels and patterns of expression in the other strains may be the result of position effects (*5*). It is also possible that expression is affected by prokaryotic vector sequences in the pRSV-CAT construct. Such sequences have recently been found to have the propensity of interfering with expression of inserted genes (*9*).

The level of CAT activity in the leg muscle of strain 3 was high. CAT enzyme purified from *Escherichia coli* has a specific activity of about 100 U/mg (*10*). The CAT activity (0.12 U/mg) in the leg muscle of strain 3 indicates that the CAT enzyme constituted approximately 0.1 percent of the soluble protein of that tissue.

To determine whether CAT gene expression was being regulated at the level of transcription, we isolated total RNA from various tissues of strain 3, and determined the concentrations of CAT RNA by RNA dot blot hybridizations (Fig. 2). The amounts of CAT-specific RNA paralleled those of the CAT enzyme in the tissues that were assayed. We assumed that messenger RNA (mRNA) constitutes 2 to 4 percent of the total leg muscle RNA; thus the CAT-specific RNA (at 120 pg/μg) represented 0.3 to 0.6 percent of the leg muscle mRNA. This estimate corresponds well with the estimate of the CAT enzyme concentration. Regulation of CAT expression in our transgenic strains was therefore likely to occur at the level of transcription. We have not mapped the site of transcription initiation in our mice. However, in cultured mammalian cells containing the pRSV-CAT construct, initiation of transcription occurs at the predicted site within the RSV-LTR (*7*).

All data on CAT expression described so far were obtained with adult mice (5 to 8 months of age). In all but one of the strains, we did not detect CAT activity at embryonic stages of development. The exception was the high expressor strain 3. Low levels of activity (of the order of 1 to 3 μU per milligram of protein) were detected in whole embryos of this strain as early as day 12 of gestation. When the feet of newborn mice were assayed for CAT activity, we noted that CAT expression remained at the same low level until at least 7 days after birth. However, by adulthood, a dramatic increase in CAT expression (20,000 μU per milligram of protein) had taken place. It appears, therefore, that high-level RSV-CAT expression in transgenic mice is initiated well after birth. A similar pattern of gene activation has been observed in certain strains of mice carrying inserts of the Moloney leukemia virus genome (*11*).

In two of the nine strains of mice carrying the pRSV-CAT gene, developmental abnormalities were observed that co-segregate with the pRSV-CAT gene insert. Since seven of the strains appeared to be developmentally normal in every respect, the observed anomalies were probably due to disruptions of the genome upon integration of the foreign DNA sequences. Strain 5 was characterized by embryonic lethality: the development of roughly one half of the embryos of $F_0 \times$ wt or $F_1 \times$ wt matings was arrested before day 8 of gestation. Of the surviving progeny, at least 50 percent carried the RSV-CAT gene and also produced small litters. This case is different from the reported insertional inactivations of essential genes (*12*) in that the observed embryonic lethality was not recessive but dominant, and yet some offspring survived to pass on the trait to subsequent generations. One possible explanation for these unusual genetics is that chromosomal rearrangements accompanied

Table 1. Copy number and CAT activity (in microunits per milligram of protein) in transgenic RSV-CAT strains. Transgenic mice were generated as described (*16, 21*). Embryos were isolated from superovulated NIH FVB/N females that had been mated to males of the same strain. Supercoiled pRSV-CAT DNA (3 μg/ml) or pRSV-CAT DNA that had been linearized by restriction enzyme digestion in the pBR322 sequences (1 μg/ml) was microinjected into one of the pronuclei 15 to 16 hours after the mice were mated. Transgenic newborns were identified by isolation of tail nucleic acids and subsequent dot blot hybridization (*16, 22*) to ^{32}P-labeled pRSV-CAT. A total of 115 embryos were injected, yielding 35 newborns, 10 of which were transgenic. Nine of the transgenic mice survived to adulthood. RSV-CAT copy number in F_1 animals was determined by dot blot hybridizations to ^{32}P-labeled pRSV-CAT. Positive dots were identified by autoradiography and then were cut out and counted in a liquid scintillation counter. Copy number per haploid genome was estimated by comparison to standards that contained known amounts of pRSV-CAT plasmid DNA mixed with normal mouse DNA. CAT activity in leg muscle homogenates of representative F_1 mice was assayed and defined as described in the legends of Table 2 and Fig. 1. Percentage transmission was determined by screening the offspring of $F_0 \times$ wt matings by means of dot blot hybridizations of tail DNA.

Strain	Copy number	CAT activity	Transmission (%)
1	13	1,800	50
2	11	27,000	62
3	2	120,000	50
4	6	5,500	14
5	180	600	91
6	50	<1	29
7	0.6	<1	57
8	0.4	<1	44
9	31	<1	36

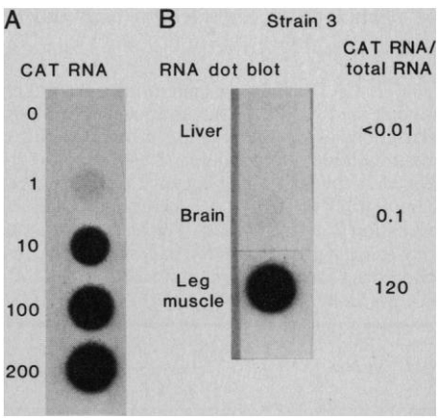

Fig. 2. CAT RNA in liver, brain, and muscle. RNA was extracted from mouse tissues as described (*19*). RNA samples were transferred to a Zetabind membrane (AMF Cuno) and probed for CAT RNA (*20*). The probe was a ^{32}P-labeled Hind III–Bam HI fragment of pRSV-CAT, which contains only CAT-coding sequences. (A) Wild-type mouse liver RNA (2 μg) plus 0, 1, 10, 100, or 200 pg of CAT RNA. The CAT RNA was made by transcription in vitro of an SP6 clone. (B) Ratio of CAT RNA (picograms) to total RNA (2 μg) from liver, brain, and skeletal muscle of an F_2 mouse of strain 3. CAT RNA in the respective organs was measured by counting each dot in a liquid scintillation counter and comparing the radioactivities to those of the CAT standards. No hybridization was seen when tissue RNA's were predigested with ribonuclease A.

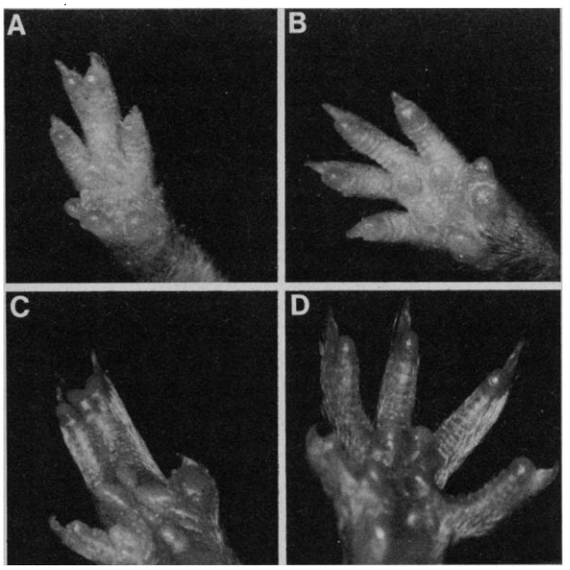

Fig. 3. Feet of F_2 mice of strain 2. (A) Front foot and (C) hind foot of an F_2 mouse of strain 2, showing the fused toe phenotype. (B) Front foot and (D) hind foot of an apparently normal sibling.

the integration of the foreign DNA. Chromosomal translocations were indeed observed in strain 5 (*13*). Mice balanced for the translocation were viable. Matings of these balanced mice to wild-type mice produced monosomy or trisomy of the translocated sequences in 50 percent of the embryos. Both monosomy and trisomy appeared to be incompatible with normal development.

In a different strain, strain 2, animals with fused toes in the fore and hind feet (Fig. 3) were observed in 3 of 15 offspring of an $F_1 \times F_1$ mating. When mice with the fused toe phenotype were mated to each other, 100 percent of their offspring showed the same anomoly, implying that these mice are homozygous for a recessive mutation associated with the integration of the foreign DNA. Woychik and co-workers (*14*) have also described a limb deformation resulting from the insertion of a foreign gene.

CAT activity was found predominantly in leg and abdominal muscle, in the heart, the sternum, tail and feet, organs rich in muscle, bone, and connective tissues. These very tissues are also targets of oncogenesis caused by RSV infection (*2*). The only genetic information common to the RSV genome and to our RSV-CAT gene construct are the terminal sequences of the viral genome. We are tempted to speculate that these sequences are responsible at least in part for target selection, all the more so because we know that the LTR's of retroviruses contain transcriptional control elements that play an important role in viral oncogenesis (*15*). It follows that sarcoma formation does not necessarily occur because mesenchymal tissues are more susceptible to transformation by the RSV *src* oncogene; instead, they may simply produce much higher levels of *src* products than other tissues.

Information targeting expression of integrated RSV-CAT sequences to specific tissues may, of course, not be restricted to the LTR domain. However, constructs containing different promoter-enhancer sequences 5′ of the CAT gene and the SV40 splice and polyadenylation signals display distinct patterns of temporal and spatial CAT expression in transgenic mice (*16, 17*).

From a practical standpoint, the RSV LTR appears to be a useful sequence to fuse to genes intended for expression in transgenic mice because the CAT signal in our RSV-CAT strains is much stronger than in strains carrying other control sequences 5′ of the CAT gene (*16, 17*). Moreover, fusion constructs involving the RSV LTR may be expected to be expressed at high levels and in specific organs during the adult life of the mouse only. This should be of particular importance for experiments involving gene products that are deleterious to the animal.

Table 2. CAT activity (in microunits per milligram of protein) in various tissues of mouse strains carrying the RSV-CAT chimeric gene. Tissue homogenates of F_1 animals were assayed for CAT activity as described in the legend of Fig. 1. In measuring enzymatic activity, the radioactive spots were localized by autoradiography, cut out, and counted in a liquid scintillation counter. One microunit of activity was defined as the amount of enzymatic activity catalyzing acetylation of 1 pmol of chloramphenicol per minute at 37°C (*10*) or 1.5 percent acetylation per hour under our assay conditions. The time of incubation was 1 to 4 hours. The amount of protein per assay was adjusted to give 40 percent or lower acetylation. Standard assays had shown that enzymatic activity was linear with time under these conditions. Each assay was adjusted to 50 μg of total protein by addition of bovine serum albumin. ND, not done.

Tissue	CAT activity in strain				
	1	5	2	3	4
Leg muscle	1,800	600	27,000	120,000	5,500
Abdominal muscle	740	ND	13,000	62,000	1,500
Foot	160	1	3,000	20,000	12,000
Tail	32	70	625	160	22,000
Sternum	240	70	11,000	25,000	620
Heart	2	1	5,700	280	2,900
Ear	1	1	100	1,100	240
Serum	<0.6	<0.6	184	7	<0.6
Spleen	<0.6	<0.6	300	56	1
Lung	1	<0.6	46	58	1
Brain	<0.6	<0.6	150	270	3
Thymus	<0.6	<0.6	12	50	14
Eye	<0.6	<0.6	16	154	12
Kidney	2	<0.6	50	110	<0.6
Tongue	2	1	155	190	35
Intestine	<0.6	<0.6	170	13	46
Liver	<0.6	<0.6	38	37	6
Testis	<0.6	ND	32	206	10

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Autolytic Processing of Dimeric Plant Virus Satellite RNA

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Associated with some plant viruses are small satellite RNA's that depend on the plant virus to provide protective coat protein and presumably at least some of the proteins necessary for satellite RNA replication. Multimeric forms of the satellite RNA of tobacco ringspot virus are probable in vivo precursors of the monomeric satellite RNA. Evidence is presented for the in vitro autolytic processing of dimeric and trimeric forms of this satellite RNA. The reaction generates biologically active monomeric satellite RNA, apparently is reversible to form dimeric RNA from monomeric RNA, and does not require an enzyme for its catalysis.

THE KNOWN SMALL SATELLITE RNA's have 400 or fewer nucleotide residues in their simplest form and have been discovered in association with the members of five groups or potential groups of RNA plant viruses (*1, 2*). They replicate detectably only when co-inoculated with the respective supporting virus. Small satellite RNA's become encapsidated in coat protein specified by the supporting virus but, with two exceptions, have no extensive nucleotide sequence homology with the virus RNA (*2*). The satellite RNA of tobacco ringspot virus (STobRV RNA) was the first small satellite RNA to be discovered (*3*). In mixed TobRV plus STobRV RNA infections, more than 90 percent of the encapsidated RNA may be STobRV RNA.

Unencapsidated, double-stranded STobRV RNA from infected tissue is composed of linear and circular multimeric forms of both polarities (*4, 5*), whereas encapsidated STobRV RNA is linear and of one polarity, designated arbitrarily as (+). The encapsidated (+)RNA is principally in the monomeric form (359 nucleotide residues), with decreasing amounts of the dimer and each of the succeeding, repetitive-sequence, multimeric forms. Observations of multimeric linear and circular forms of satellite RNA's and (the independently replicating) viroid RNA's and of enzymes capable of circularizing RNA (*5–9*) have led to the formulation of replication models that include rolling-circle transcription and the processing of multimeric transcripts to form the monomeric RNA (*4, 8, 9*).

We studied two independent isolates of STobRV RNA, designated budblight and NC-87 (*10*). Virus particles (*11*) from leaves of infected common bean (*Phaseolus vulgaris* cv. Black Valentine) were chromatographed on 2 percent agarose beads (Sepharose CL-2B, virus particle distribution constant ≈0.6) in 10 m*M* NaH_2PO_4, 2 m*M* EDTA, and 0.25*M* NaCl (*p*H ~6.5; column linear flow rate, 0.6 mm/min). RNA was isolated by a two-phase phenol extraction procedure (*11*), and purification of monomeric, dimeric, and trimeric forms of the satellite RNA was by electrophoresis at 30 volt/cm through 0.5 mm thick, 6.5 percent polyacrylamide gels in 7*M* urea, 90 m*M* tris-borate, and 1 m*M* EDTA buffer (*p*H 8.3) (*12, 13*).

Electrophoresis of purified dimeric and trimeric STobRV RNA preparations gave single zones of the expected mobilities, but only if the RNA was analyzed directly after isolation (Fig. 1b). After storage of RNA at

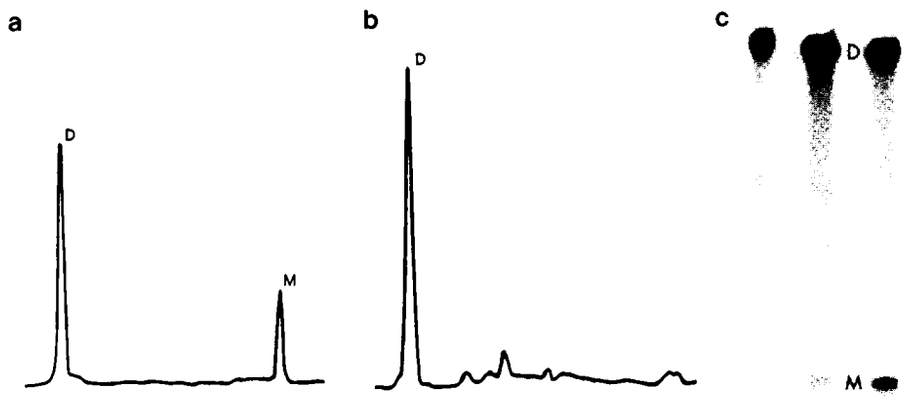

Fig. 1. Formation of RNA with the electrophoretic mobility of monomeric STobRV RNA from the dimeric form. After incubation in buffered solution as indicated below, the samples were heated to 80°C for 30 seconds in 50 percent formamide containing an excess of EDTA over divalent metal ion. Electrophoresis was at 350 V in 10 cm long, 7 percent polyacrylamide gel (a and b) or at 1500 V in 40 cm long, 6.5 percent polyacrylamide gel (c), both containing 7*M* urea. NC-87 strain dimeric STobRV RNA, when incubated in 33 m*M* tris, 5.5 m*M* HCl, 10 mg of SDS per milliliter, and 20 m*M* $MgCl_2$ (*p*H ~7.4) for 15 minutes at 30°C generated a zone with the mobility of the monomeric STobRV RNA from virus particles (a), whereas the control incubation with 2 m*M* EDTA in place of $MgCl_2$ did not (b). Detection was by silver stain (*18*) and optical densitometry. The sample for (c) was dimeric budblight strain STobRV RNA partially 5′ end-labeled (less than 5 percent of the molecules derivatized) by incubation with bacteriophage T4 polynucleotide kinase and [γ-^{32}P]ATP. The dimeric RNA was incubated in 100 m*M* tris, 45 m*M* HCl, 10 mg of lithium dodecyl sulfate (LDS, *p*H 8.0) per milliliter, and either 50 m*M* EDTA on ice or at 25°C (left-hand and central lanes, respectively) or 5 m*M* $MgCl_2$ at 25°C (right-hand lane) for 30 minutes before electrophoresis and detection by autoradiography. D and M indicate the zones for dimeric and monomeric STobRV RNA, respectively.

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