Specificity of Initiation of Plus-Strand DNA by Rous Sarcoma Virus

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We previously reported that in the endogenous reaction of Rous sarcoma virus disrupted by melittin, plus-strand DNA initiates on a small oligonucleotide primer and that this initiation can be reconstructed in vitro in reactions containing purified minus-strand DNA as template, viral RNA as a source of primer, and reverse transcriptase (Smith et al., J. Virol. 49:200–204, 1984). Further studies on the specificity of initiation in the endogenous reaction have shown the following. (i) The primer was 12 nucleotides in length. Its sequence began with a 5′ pyrimidine, followed by 11 purines, ending with rGra-3′. This sequence was in agreement with the known plus-strand RNA sequence immediately upstream from the initiation site. Thus, the primer began one nucleotide 5′ to the so-called polyuridine tract that has been found on all retrovirus genomes. (ii) The transition point between RNA primer and DNA product was precisely located. It was before the end of the polyuridine tract. Thus the polyuridine tract, although essential for virus replication and probably a flag for the priming event, did not define the limits of the RNA primer. After primer removal, the DNA had a 5′ phosphate, consistent with generation by the viral RNase H activity. The priming specificity in reconstructed reactions was also examined further, with the following observations. (i) When the source of RNA primer was prehybridized to the template viral DNA, the generation, utilization, and subsequent removal of primer were essentially the same as those observed in the endogenous reaction. In the absence of deliberate prehybridization, some specific activity was lost. There were additional locations for the 5′ end of the primer as well as the transition point between RNA primer and DNA. (ii) Purine-rich oligoribonucleotides created by RNase A digestion of viral RNA could prime strong-stop plus DNA, but again with the loss of specificity relative to that in the endogenous reaction. (iii) The 5′ end of the minus-strand DNA template was not required for initiation of strong-stop plus DNA. Therefore, the specificity of initiation did not depend upon an intramolecular interaction requiring the two inverted repeat sequences that flank the long terminal repeat.

During the replication of retroviruses a viral enzyme, reverse transcriptase, copies the viral RNA genome into a double-stranded DNA intermediate (reviewed in reference 27). Minus-strand DNA (complementary to the viral RNA genome) is synthesized first, using a cellular tRNA as the primer (5). The growing minus-strand DNA is the template for the synthesis of plus-strand DNA. The first plus-strand DNA seen is a discrete species called strong-stop plus DNA (14, 29). Strong-stop plus DNA is initiated at a precise location (13). For avian sarcoma virus, this species is ca. 340 nucleotides in length (29).

We previously demonstrated that in the endogenous reaction of avian sarcoma virus virions permeabilized with melittin, the strong-stop plus DNA is initiated on an oligoribonucleotide primer (21). The specific initiation is the result of three precise nucleolytic events: two to generate the discrete ends of the primer and a third to remove the primer after use.

We also showed that strong-stop plus initiation can be reconstructed with purified components: minus-strand DNA as template, high-molecular-weight viral RNA as a source of primers, and reverse transcriptase (21). Champoux et al. (3) have used similar reconstructed reactions to show that RNase A-digested viral RNA can provide primers for the initiation of strong-stop plus DNA by Moloney murine leukemia virus.

It has been suggested that degradation of the viral RNA by RNase H, an activity of the reverse transcriptase, could provide primers for plus-strand DNA synthesis (27, 29). Such a mechanism has been demonstrated in the reverse transcription of homopolymers (31). In addition, RNA-DNA junctions have been detected in the total plus-strand DNA synthesized in a reconstructed reaction (17). Our data on the specificity of initiation, as presented here, are consistent with such a role for RNase H.

In all retroviruses examined, the initiation site of strong-stop plus DNA is adjacent to a series of purine nucleotides in the viral RNA (28). This polyuridine tract (PPT) is required in cis for retroviral replication (22). Thus the circumstantial evidence is that the PPT plays a role in the priming of plus-strand DNA synthesis. Our studies have determined the actual relationship between this PPT and the RNA species used as the primer.

MATERIALS AND METHODS

Virus, cells, and reagents. The Prague A strain of Rous sarcoma virus was derived from the transformed quail clone Q-Pra-4 (19). The virus was propagated in quail embryo fibroblasts as previously described (7). Restriction enzymes were used according to the instructions of the manufacturer (Bethesda Research Laboratories), with the addition of 0.5 U of Ribonuclease (Bolton Biologicals) per μl. Reverse transcriptase, purified from avian myeloblastosis virus, was generously provided by the Office of Program Resources and Logistics, Virus Cancer Program, Bethesda, Md.

Plasmid pSRA2, which contains Rous sarcoma virus DNA (Schmidt-Ruppin A strain) inserted into pBR322, was obtained from W. DeLorbe and co-workers (4). DNA synthesis and analysis. Conditions for the synthesis and analysis of strong-stop plus DNA from endogenous reactions and from reconstructed reactions were as previously described (21) and are presented briefly here.

In the endogenous reactions, pelleted virions were permeabilized with melittin and incubated with deoxyribonucle-
otide triphosphates. Labeled DNA products were made by including [α-32P]TTP in the reaction mixture. After digestion with either EcoRI or SphI, the DNA product was denatured and hybridized in solution to an excess of DNA from a recombinant M13 bacteriophage which contained the minus strand of the long terminal repeat (LTR) (25). The hybrid molecules were isolated by rate zonal sedimentation. This provided a partial purification of those labeled molecules containing sequences related to strong-stop plus DNA. This DNA was then analyzed on an 80-cm gel of 6% acrylamide-urea. A cloned fragment of DNA which contained the origin of strong-stop plus DNA (an EcoRI-PvuII fragment of pSRA2) was submitted to base-specific cleavages described by Maxam and Gilbert (11) and served as a size standard on the high-resolution acrylamide gels.

For the reconstructed reactions we used as a template minus-strand DNA synthesized in an endogenous reaction. The higher-molecular-weight DNA species were isolated by two cycles of rate zonal sedimentation through alkaline sucrose. This DNA was incubated with viral RNA and reverse transcriptase. Labeled DNA products were made by the addition of [α-32P]TTP to the reaction. Labeled species related to strong-stop plus DNA were partially purified and then analyzed by gel electrophoresis as described above. Unlabeled reaction products were glyoxalated (12) and subjected to electrophoresis on slab gels of 2% agarose. After transfer to nitrocellulose (23), sequences related to strong-stop plus or its complement were detected with probes made from recombinant M13 DNAs containing inserted LTR sequences (9, 25).

RESULTS

Size and sequence of the RNA primer in endogenous reactions. In our previous studies, the RNA primer on plus-strand DNA appeared to be uniform in size (21). The following studies were undertaken to determine whether the primers were predominantly of a single sequence and, if so, to compare that sequence to the published sequence of the viral DNA (20). To do this we purified those labeled species from an endogenous reaction that were related to strong-stop plus DNA. A map of the DNA around the origin of strong-stop plus and the locations of the restriction sites used in this study are shown in Fig. 1. This DNA was digested with EcoRI and analyzed on an acrylamide gel (Fig. 2a, lane 2). As described previously (21), species C and D correspond to the EcoRI fragments of strong-stop plus DNA without or with the 18 nucleotides copied from the minus-strand tRNA primer. The labeled band which is one nucleotide larger than band D represents strong-stop plus DNA in which only a single base has been copied from the tRNA primer. Species A and B correspond to the 5′ EcoRI fragments with or without the oligoribonucleotide primer. Relative to the single-nucleotide calibration provided by the adjacent sequencing ladder, the primer was reproducibly measured to be 12 bases in length (Fig. 2; unpublished data). To confirm this size assignment and to characterize the sequence of the primer, samples were digested to completion with base-specific RNases. RNase A, which cuts 3′ to pyrimidines, reduced the primer length by one nucleotide (Fig. 2a, lane 1). RNase T1, which cuts 3′ to G, reduced the primer to one nucleotide in length (Fig. 2a, lane 3). RNase U2, which cuts 3′ to A, and alkali, which hydrolyzes all ribonucleotides, both removed the entire primer (Fig. 2a, lanes 4 and 5, respectively). From these digestions the sequence of the RNA primer was deduced to be 5′-Y(R)GA-3′ where Y = T or C and R = A or G. This sequence is consistent with the known RNA sequence of the viral RNA (20) immediately upstream from the initiation site.

![Diagram of nucleotides](image-url)
Removal of the RNA primer in endogenous reactions. To examine the 5' terminus more closely, the strong-stop plus DNA was digested with Sph I. This enzyme cuts ca. 100 bases from the 5' end (Fig. 1), releasing fragments A' and B', which are smaller and hence better resolved than the corresponding fragments (A and B) released by EcoRI (Fig. 2b, lane 1). The migration of the fragments was measured by scanning the autoradiograms with a densitometer and then measuring the distance between the various peaks relative to the single nucleotide spacing. When the sample was first treated with alkali, band A' disappeared, as expected, and a novel species appeared which migrated slightly slower than band B' (Fig. 2b, lane 2). The difference in migration relative to B' was equivalent to one third of a single nucleotide increment. The identity of the novel species became apparent when the sample was treated with alkaline phosphatase before electrophoresis (Fig. 2b, lane 3). Band B' then disappeared, and only the novel species remained. We interpreted these results to mean that the natural removal of the primer in the endogenous reaction leaves a 5' phosphate on strong-stop plus DNA. In contrast, removal of the primer with alkali leaves a 5' OH, resulting in a species with a relatively slower electrophoretic mobility. The ability of a terminal phosphate to increase mobility has been previously described (24). RNase H is known to produce oligoribonucleotides with a 5'-terminal phosphate (2, 30). This experiment showed that the strong-stop plus DNA also has a 5' phosphate after primer removal.

Specificity of initiation in reconstructed reactions. Studies were undertaken to compare the specificity of priming and plus-strand initiation observed in endogenous reactions to that obtained in reconstructed reactions with purified components: reverse transcriptase, minus-strand DNA as template, and viral RNA as a source of primers. The products from such reconstructed reactions, with or without alkali treatment to remove the primer, are shown in Fig. 3, lanes 1 and 2. Similar to the products of endogenous reactions, we observed the bands previously designated A, B, and D. Band C was absent. This was expected since in the endogenous reaction band C corresponds to the 3' end of strong-stop plus DNA with an additional 18 nucleotides copied from the minus-strand tRNA primer. The minus-strand template used in the reconstructed reactions had been treated with both RNase A and alkali so species C could not be made. For the products of an endogenous reaction, bands A and B were homogeneous (Fig. 2). However, for the reconstructed reactions a size heterogeneity was observed in both bands A and B (Fig. 3, lanes 1 and 2). There were a different number of extra species around band A as compared with band B, and we interpreted this to mean that the heterogeneity was due to differences in both the size of the RNA primer and the position of the RNA-DNA junction. These heterogeneities were significantly reduced in reconstructed reactions in which the template DNA and source of RNA primers were hybridized before synthesis (Fig. 3, lanes 3 and 4).

We have previously shown that an RNase A digest of viral RNA is an efficient source of primers for the synthesis of strong-stop plus DNA in reconstructed reactions (21). This was expected since RNase A only cleaves at pyrimidines and since the sequence upstream from the initiation site of strong-stop plus DNA is made up of purines, the PPT. Figure 4d shows the RNA oligomer that would be generated from an RNase A digestion of viral sequences around the strong-stop plus DNA origin. Further experiments were undertaken to characterize the RNA species that were actually used as primers and to determine where the plus-strand DNA was initiated in such a reconstructed reaction (using RNase A-digested viral RNA as a source of primers) relative to an endogenous reaction. As before, labeled strong-stop plus-containing DNA products were isolated, digested with EcoRI, and subjected to electrophoresis on polyacrylamide gels without or with a previous alkali treatment (Fig. 3, lanes 5 and 6). The resulting bands were compared to an adjacent sequencing ladder of cloned DNA, as in Fig. 2A. The indicated band B in lane 6 corresponds to the single DNA initiation site, after primer removal, as seen in the endogenous reactions (Fig. 2a). Clearly in this reconstructed reaction DNA synthesis initiated at two additional sites, apparently located one nucleotide on either side of that site. The indicated band A in lane 5, with RNA primer still associated, showed the same degree of heterogeneity as band B (Fig. 3). We interpreted this as evidence that the primers were predominantly of a single size, 11 bases, and that the heterogeneity around bands A and B was due to two new positions for the location of the RNA-DNA junction. A comparison of lanes 5 and 6 also suggests that RNase H removes the RNA primers more efficiently from incorrectly initiated molecules than from those initiated at the correct location (Fig. 3). The significance of this finding is being further investigated.

Role of the template DNA in specific plus-strand initiation. In the above studies with endogenous reactions, and even with reconstructed reactions, the size of the RNA primers and the location of the RNA-DNA junction were remarkably specific. The following experiment was undertaken to test an hypothesis of how the viral minus-strand DNA template might contribute to this specificity.

The viral LTR is essentially a double-stranded copy of

FIG. 3. Acrylamide gel electrophoresis of strong-stop plus DNA from reconstructed reactions. Minus-strand viral DNA containing 0.6 ng of LTR minus-strand sequences was incubated in a reaction mixture containing reverse transcriptase and viral RNA as indicated. Labeled products related to strong-stop plus DNA were enriched for and analyzed on acrylamide gels as described in the legend to Fig. 2. Lanes: 2, 180 ng of viral RNA (containing 6.6 ng of LTR sequences) added; 1, sample as in lane 2 except treated with alkali (0.3 N NaOH; 100°C; 10 min); 3, 180 ng of viral RNA added, prehybridized (to a c/s of 1 mol · s/liter) to DNA template; 4, sample as in lane 3 except treated with alkali; 5, 180 ng of viral RNA added, predigested with RNase A; 6, sample as in lane 5 except treated with alkali. Bands labeled A and B are as described in the text, and the dots represent single nucleotide spacings from a sequencing ladder run in an adjacent lane, as in Fig. 2.
strong-stop plus DNA. It is flanked by a short inverted repeat sequence (IR) which for Rous sarcoma virus is 15 bases in length with three mismatches (20). Thus on the minus-strand DNA template, a copy of the IR is located precisely at the initiation site of strong-stop plus DNA. A copy complementary to this is present at the 5' end of the template (Fig. 1). This suggested that a possible mechanism for the specific primer generation and utilization might involve an interaction between these two IR elements. To test this hypothesis we digested a sample of endogenously synthesized DNA with HpaII before purifying the fragment containing the origin of strong-stop plus DNA on an alkaline sucrose gradient. Such a digestion should remove the 5' terminus of the minus-strand template, including one copy of the IR (Fig. 1). We compared the activity of this DNA template relative to undigested minus-strand template in reconstructed reactions. Unlabeled reaction products were analyzed on agarose gels. After Southern transfer (23), LTR-specific probes were used to detect template and product DNA (Fig. 5). The three lanes labeled (−) are from reconstructed reactions with the undigested minus-strand template, and those labeled (+) are from reactions with the HpaII-digested template. An M13 probe specific for LTR minus-strand sequences was used to show the size of the minus-strand DNA template. The undigested DNA template was large and heterogeneous, but after HpaII digestion was reduced to a discrete band of 850 bases, the predicted size of the HpaII fragment-containing LTR sequences (Fig. 5, lanes 1). Lanes 2 and 3 show what was detected with an M13 probe specific for LTR plus-strand sequences in reconstructed reactions with no added primers (lanes 2) or in reactions with viral RNA added as a source of primers (lanes 3) (Fig. 5). The main plus-strand product from the undigested template was strong-stop plus DNA, whereas the major product from the HpaII digested template was 260 bases (Fig. 5, lanes 3). This was the size predicted for correct initiation of strong-stop plus DNA and for elongation to the HpaII site. In summary, this experiment did not detect any role for an interaction between the IR sequences in the specific initiation of strong-stop plus DNA.

**DISCUSSION**

These studies have shown that in the synthesis of strong-stop plus DNA in the endogenous reaction of disrupted virions several remarkably specific events are involved (Fig. 4b and c). (i) Only one size of primer was seen, indicating precise 5' and 3' ends. (ii) The site of initiation of DNA was always at the same nucleotide. (iii) The primer was apparently removed in a single step, since intermediates containing a partial primer were not detected. Furthermore, the sequence of the primer was both uniform and consistent with the RNA sequence (20) expected upstream from the origin of strong-stop plus DNA.

These specificities of the endogenous reaction were also achieved in reconstructed reactions with purified components. The necessary specificities thus could be provided by the template DNA, the source of RNA primers, and reverse transcriptase. Apparently there was no requirement for cellular or additional viral factors, such as specific nucleic acid binding proteins. This does not exclude the possibility that in vivo other factors could play an auxiliary role. The observed in vitro use of RNA primers, in the absence of ribonucleotide precursors, has also ruled out de novo synthesis of primers, for example, by a primase.

The combined evidence indicates that the RNA primer is generated from the viral RNA by the action of the RNase H activity present in the viral reverse transcriptase. The main argument is that it was the only nuclease available in the reconstructed reaction. RNase H degrades the RNA portion of DNA-RNA hybrids (15). The avian retrovirus RNase H generally releases oligoribonucleotides of 10 to 12 bases with a 5'-terminal phosphate (2, 30), in agreement with our observations. However, the documented properties of the viral RNase H are not sufficient to explain the specificities we have observed. It may be that RNase H is more specific than has been previously described, or additional factors may be involved. One such factor would be that we assay the functional combination of primer generation and primer utilization; RNase H must generate many other oligonucleotides that fail to act as primers. Another factor could be the primary structure of the template. As others have noted, every retroviral genome contains a PPT in this region (28), and it is essential for infectivity (22). For another "retroid virus", cauliflower mosaic virus, there are two specific sites for plus-strand DNA initiation, and both have a PPT in the immediate vicinity (6). Nevertheless, it is important to stress that the PPT may have other roles, and as far as the initiation of plus-strand DNA synthesis, the PPT is at most a flag. It is
In summary, a variety of factors seem to influence the specific initiation of viral plus-strand DNA, but in vitro at least, all the factors are contributed by the template, primer, and enzyme. This is in marked contrast to the multicomponent systems known to be required for correct initiation of procaryotic genomes and bacteriophages (8).

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LITERATURE CITED


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