Transcription of cloned Moloney murine leukemia proviral DNA injected into Xenopus laevis oocytes

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ABSTRACT

We have microinjected genomic DNA clones containing the Moloney murine leukemia virus (M-MuLV) proviral genome and flanking mouse sequences from Mov-3, Mov-7 and Mov-10 mice into Xenopus laevis oocytes and analyzed the virus-specific transcription and translation products. These mouse strains carry a proviral genome copy of M-MuLV in their germ line at different chromosomal positions and differ from each other with respect to expression of the proviral genome. We show here that the different M-MuLV proviral genome copies were transcribed into virus-specific RNA with similar efficiencies. Transcription of viral RNA initiated correctly at the viral promoter in the 5' LTR, whereas the promoter in the 3' LTR was used with a much lower frequency, if at all, for initiation of RNA synthesis. Most of the virus-specific transcripts were smaller than authentic M-MuLV mRNA and confined to the oocyte nucleus. Using immunoprecipitation, we were not able to detect virus-specific proteins after injection of proviral DNA, whereas after injection of a comparable amount of M-MuLV mRNA viral protein was readily detectable.

INTRODUCTION

Two kinds of assay systems have been developed in the past to study the regulation of eukaryotic gene expression on the transcriptional level: direct in vitro transcription in cell-free extracts (1,2) and the introduction of genes into living cells by transfection (3-5) or microinjection (6). The injection of DNA into amphibian oocytes was one of the first and most widely used systems to study transcription of eukaryotic genes (7). The characteristics and advantages of this system have recently been reviewed (8). Retroviruses are suitable models for studying eukaryotic gene expression. They have been shown to contain within their genome (in the long terminal repeats, LTR) a polymerase II initiation site and the usual regulatory signals common to most
We have developed in our laboratory mouse substrains that carry a proviral genome copy of M-MuLV in their germ line at different chromosomal positions (14). These mouse strains, which have been characterized in detail elsewhere, differ from each other with respect to expression of the proviral genome: whereas some strains regularly activate infectious virus, others do not (15,16). One of our attempts to study the regulation of retrovirus transcription was to micro-inject cloned proviral genome copies from these mouse substrains into X. laevis oocytes, to characterize the virus-specific transcription and translation products, and to correlate this to the in vivo expression of the respective proviral genomes. We have used for these experiments the cloned M-MuLV proviral copies from the viremic Mov-3 mouse substrain and from the non-viremic Mov-7 and Mov-10 substrains. The results show that M-MuLV DNA from all three substrains is transcribed by the oocytes into virus-specific RNA. A further characterization of the transcription products by Northern blot analysis and Sl-mapping techniques shows that, although the oocytes correctly use the viral promoter for initiation of transcription, the transcription efficiency is rather low and only a small fraction of the virus-specific RNA is functional mRNA.

MATERIALS AND METHODS

M-MuLV Proviral DNA Clones

The mouse genomic DNA clones containing the M-MuLV proviral genome and flanking mouse sequences from Mov-3, Mov-7 and Mov-10 mice have been described elsewhere (17-19). A partial restriction map is shown in Fig. 1. Since it had been reported that pBR-DNA sequences have an inhibitory effect on eukaryotic gene transcription in X. laevis oocytes (29), most of the experiments were performed with DNA from which the plasmid sequences had been removed. To achieve this, the DNA was cleaved with EcoRI and the fragments were separated by centrifugation on 5-20% NaCl gradients. The EcoRI fragment containing the proviral genome and flanking mouse sequences was re-ligated prior to injection into oocytes. Throughout this paper the presence of plasmid sequences is marked by a p; e.g., pMov-3 DNA refers to the genomic DNA
clone containing the M-MuLV proviral genome and flanking mouse sequences from Mov-3 mice and pBR322 sequences, whereas Mov-3 DNA refers to the same clone after removal of pBR322 sequences.

**Oocyte Injection**

The handling of frogs and oocytes and the preparation of oocyte nuclei and cytoplasm was described previously (20,21). Microinjection of DNA into oocyte nuclei was done directly without centrifugation from the animal pole with micropipettes of 10-15 nm tip diameter. The DNA concentration was 0.5-1.0 ng/μl, the injected volume 10 μl. Dye-injected control oocytes showed that in 80-90% the nuclei were hit successfully. RNA was injected into the oocyte cytoplasm as described (21). When the oocytes were labeled with $^{32}$P or $^{35}$S-methionine, the label was added 24 hrs after DNA or RNA injection. The labeling periods were 24-48 hrs.

**RNA Preparation and Analyses**

Oocytes were homogenized in 10 mM Tris HCl, pH 7.5, 1 mM EDTA (approximately 30 μl per oocyte), extracted 2 x with phenol-chloroform and 2 x with chloroform, and precipitated with ethanol. DNA and carbohydrate were removed by at least one precipitation of the RNA with 2 M LiCl at 0°C overnight and a subsequent treatment with 40 μg/ml of DNase I for 30 min at room temperature. The DNase I, although declared RNase-free by the manufacturers, had to be purified by affinity-chromatography on agarose-coupled aminophenylphosphoryl-uridine-2'(3')-phosphate (22) to remove traces of contaminating RNase activity.

Quantitation of M-MuLV-specific RNA by annealing to a representative M-MuLV cDNA probe was done as described (23). Denaturation of RNA with glyoxal, agarose gel electrophoresis, transfer to nitrocellulose filters, and hybridization to M-MuLV cDNA was performed according to published procedures (24,25).

**Immunoprecipitation**

Oocytes were homogenized in 10 mM phosphate buffer, pH 7.6, containing 1 mM EDTA, 1% NP40, 1% deoxycholate, 0.3% SDS, 1 mM PMSF and 1 mM TPCK, and immunoprecipitates prepared and analyzed by polyacrylamide gel electrophoresis as described (21).

**S1 Nuclease Mapping**

A 515 bp Sau 3a fragment from the 5' LTR region of Mov-3 DNA,
Fig. 1: Partial restriction map of Mov-3 and Mov-7 DNA. A: The positions of the EcoRI restriction sites in the flanking mouse sequences of Mov-3 and Mov-7 DNA and the PvuII restriction sites within the proviral genome are shown (compare the text). B: The 5' and 3' LTRs with some restriction sites relevant for the preparation of the probes used for S1-mapping. The sites of initiation (cap-site) and termination (poly(A)-site) of transcription are indicated (arrows). The dotted arrows show the positions of the potential poly(A)-site in the 5' LTR and of the cap-site in the 3' LTR (compare the text).

A 1200 bp Clal-PstI fragment containing the 3' LTR and flanking cellular sequences of Mov-3 DNA, and an 800 bp PvuII fragment containing the 3' LTR and flanking sequences of Mov-7 DNA were used as probes (Fig. 1). To avoid a preferential labeling at the 5'-protruding Clal site, the Clal-PstI fragment was treated with S1 nuclease prior to end-labeling. 5'-End-labeling of dephosphorylated DNA with polynucleotide kinase and $\gamma^{32}$P-ATP was done as described (26).

For S1 nuclease mapping of the 5' termini of RNA, 10-50 µg of RNA and 250-500 ng of the end-labeled probe were precipitated with ethanol, dried, and resuspended in 30 µl hybridization buffer containing 80% formamide, 400 mM NaCl, 50 mM PIPES, pH 6.4, and 1 mM EDTA (27). DNA and RNA were denatured (2 min at 100°C) and re-annealed for 4 hrs or overnight at 50°C. 300 µl S1 buffer (250 mM NaCl, 10 mM NaCH$_3$CO$_2$, pH 4.6, 1.5 mM ZnSO$_4$, 5% glycerol) containing 50-100 units S1 nuclease per assay were added and the
Fig. 2: Quantitation of M-MuLV-specific RNA by Crt analysis. RNA was prepared from purified M-MuLV virions (□), M-MuLV-producing fibroblasts (●), control oocytes (○) and Mov-3 DNA-injected oocytes (○), and virus-specific sequences quantified by Crt analysis (23). (X): RNA from Mov-3 DNA-injected oocytes after alkali treatment; (▲) RNA from oocytes injected with Mov-3 DNA linearized prior to injection.

digestion was performed at 45°C for 1 hr. The samples were then phenol-chloroform-extracted, precipitated with ethanol, and analyzed on 5% polyacrylamide gels in Tris-borate buffer, pH 8.3 (26) and autoradiographed.

RESULTS
Transcription Efficiencies of Cloned Retroviral Genomes in X. laevis Oocytes

Cloned retrovirus genomes were injected into X. laevis oocyte nuclei. After 24-48 hrs of incubation, RNA was extracted from the oocytes, purified, and analyzed for M-MuLV-specific sequences by liquid hybridization with a representative M-MuLV cDNA probe (23). Fig. 2 shows that RNA prepared from oocytes injected with Mov-3 DNA hybridized with a Crt 1/2 value of 80. The hybridization was due to newly synthesized RNA rather than the injected DNA, since it could be abolished by alkali treatment of the RNA prior to
hybridization (Fig. 2). There was no significant synthesis of virus-specific RNA when the DNA was injected in a linear form, confirming results of others (29,36). We have also co-injected DNA and α-amanitin (0.01 and 0.1 ng per oocyte corresponding to 0.01 and 0.1 μg/ml oocyte volume) and found a reduction of about 50% in the transcription of virus-specific RNA at both concentrations (not shown), indicating that at least part of the RNA was the result of transcription by polymerase II. However, we have not performed a systematic study of the effect of α-amanitin on M-MuLV-specific RNA transcription. The final level of hybridization usually was about 80% and not significantly different from the level obtained with RNA from virus-producing fibroblasts (Fig. 2). This shows that transcripts representing the entire viral genome were made in the oocytes.

Using the Crt 1/2 values obtained by liquid hybridization, we calculated the relative concentrations of viral RNA in RNA preparations from oocytes injected with different DNA clones and, assuming an average RNA content of 4 μg per oocyte (ref. 8 and unpublished results), the transcriptional efficiencies of the different clones injected. The results of several experiments are summarized in Table I. It appeared that the different retrovirus clones used in these experiments (Mov-3, Mov-7, Mov-10) were transcribed with approximately equal efficiencies (0.2-0.6 ng RNA synthesized/ng DNA injected). This indicated that there was no significant influence of the flanking cellular sequences on transcription of the proviral genome. This was also shown by removal of most of the 5′ flanking mouse sequences from pMov-7 DNA without significant change in transcription efficiency (Table I). Mov-7 DNA was usually transcribed with a 2- to 5-fold higher efficiency than Mov-3 DNA. These DNAs were used for most of the experiments described in the following sections. The presence of pBR sequences in the injected clone had a 2- to 5-fold inhibitory effect on the transcription of viral sequences. The orientation of the proviral genome in the recombinant plasmid had no significant effect (Table I).

Detection of Virus-Specific Proteins Synthesized in Oocytes Injected with M-MuLV Genome Copies and mRNA

It has been shown (28-30) that genes injected into frog oocytes
Table I. Transcription Efficiencies of Different Clones Injected Into X. laevis Oocytes

<table>
<thead>
<tr>
<th>DNA</th>
<th>Crt 1/2</th>
<th>% Viral RNA</th>
<th>ng RNA/ng DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>70S RNA</td>
<td>3.6x10^-2</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>C11-1AC</td>
<td>23</td>
<td>0.16</td>
<td>-</td>
</tr>
<tr>
<td>pMov-3</td>
<td>55</td>
<td>0.07</td>
<td>0.28</td>
</tr>
<tr>
<td>Mov-3</td>
<td>26</td>
<td>0.14</td>
<td>0.56</td>
</tr>
<tr>
<td>Mov-3</td>
<td>80</td>
<td>0.05</td>
<td>0.2</td>
</tr>
<tr>
<td>Mov-10</td>
<td>120</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>pMov-7 dir.</td>
<td>60</td>
<td>0.06</td>
<td>0.24</td>
</tr>
<tr>
<td>pMov-7 opp.</td>
<td>30</td>
<td>0.12</td>
<td>0.48</td>
</tr>
<tr>
<td>pMov-7 part.</td>
<td>28</td>
<td>0.13</td>
<td>0.52</td>
</tr>
<tr>
<td>Mov-3</td>
<td>32</td>
<td>0.11</td>
<td>0.44</td>
</tr>
<tr>
<td>Mov-7</td>
<td>70</td>
<td>0.05</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.14</td>
<td>0.56</td>
</tr>
</tbody>
</table>

a pMov-3 and Mov-3 DNA refer to DNA with and without pBR322 sequences, respectively (compare the text).
b pMov-7 dir. and pMov-7 opp. contain the Mov-7 DNA in opposite orientations; pMov-7 part. is a clone from which most of the 5' flanking cellular sequences had been removed.

are transcribed into functional mRNA which can be expressed as protein. To see whether viral proteins were synthesized, we labeled the oocytes 24 hrs after injection of M-MuLV DNA with ^35S-methionine for a period of 24 hrs and analyzed the proteins after immunoprecipitation with anti-M-MuLV serum. The results of these experiments are summarized in Fig. 3. Most attempts to demonstrate virus-specific proteins synthesized by the frog oocytes after injection of M-MuLV DNA clones failed. Only in 1 of 4 experiments did we detect a protein with a molecular weight of approximately 65K, which could be competed out by addition of cold viral protein (Fig. 3d). This protein therefore may represent the gag polyprotein pr65^gag or the unglycosylated precursor of the env-protein (31-33). To determine whether M-MuLV mRNA could induce synthesis of virus-specific proteins under our experimental condi-
Fig. 3: Analysis of M-MuLV-specific proteins by immunoprecipitation. Proteins from $^{35}$S-methionine labeled M-MuLV-producing fibroblasts (a), M-MuLV virions (b) and oocytes (c-i) were immunoprecipitated with anti-M-MuLV serum in the absence (1) or presence (2) of excess cold viral protein and analyzed by electrophoresis on polyacrylamide gels. The oocytes were injected with Mov-3 DNA (c,g), Mov-7 DNA (d,h), poly(A)$^+$ RNA from M-MuLV-producing fibroblasts (f) or uninjected (e,i). m = Marker proteins.

...tions, we injected into the oocytes a comparable amount of poly(A)$^+$ RNA from M-MuLV-producing fibroblasts. Fig. 3f shows a strong band of the expected size, whereas this protein was not detectable in the oocytes injected with DNA in the same experiment (lanes g and h). From the Crt 1/2 values (compare Table I) it was possible to estimate that the absolute amount of virus-specific RNA in both cases was between 2 and 6 ng per oocyte. Mixing experiments with immunoprecipitates from mRNA-injected and control oocytes showed that our method was sensitive enough to detect 10 x less virus-specific proteins (not shown). This result therefore suggests that most of the virus-specific RNA synthesized in the oocytes after injection of M-MuLV DNA was not functioning as mRNA.

Characterization of Virus-Specific Transcripts

To characterize the RNA transcribed from the injected DNA, we labeled the oocytes with $^{32}$P and hybridized the labeled RNA to M-MuLV DNA restriction fragments (see Fig. 1). After digestion
Fig. 4: Analysis of M-MuLV-specific RNA from injected oocytes by hybridization to M-MuLV DNA restriction fragments. Mov-3 DNA, Mov-7 DNA, or no DNA (ctr) were injected into X. laevis oocyte nuclei. Twenty-four hrs later \( ^{32} \text{P}-\text{UTP} \) was injected. After additional 24 hrs RNA was prepared and hybridized to (a) salmon sperm DNA, (b) EcoRI-cleaved and (c) PvuII-cleaved pMov-3 or pMov-7 DNA, respectively. The hybrids were digested with SI nuclease, separated by agarose gel electrophoresis and visualized by autoradiography. The arrow indicates a size marker of 8.8 kb, the length of the M-MuLV genome.

with SI nuclease the hybrids were analyzed by agarose gel electrophoresis and autoradiography. When EcoRI fragments were used for the hybridization, we found heterogenous hybrids ranging in size from more than 8.8 kb to less than 1 kb; with PvuII fragments we also found a heterogenous distribution of hybrids showing, however, a main band of approximately M-MuLV genome length and several smaller subbands (Fig. 4). These results indicate that transcripts from the entire viral genome as well as from flanking cellular sequences were produced in the oocytes after injection of Mov-3 or Mov-7 DNA. However, they do not allow any conclusions concerning the size of the transcripts. Since it is known that SI nuclease cleavage of a phosphodiester band opposite a nick is inefficient, the RNA-DNA hybrids of more than M-MuLV genome size (Fig. 4, lanes b) or the 8.8 kb band (Fig. 4, lanes c) are not
Fig. 5: Analysis of M-MuLV-specific RNA by Northern blot analysis. Oocytes were injected with pMov-3 DNA (lanes 2), Mov-7 DNA (lanes 3) and Mov-3 DNA (lanes 4). After 36 hrs nuclei and cytoplasm of 10 oocytes each were separated and RNA extracted and analyzed by gel electrophoresis, transfer to nitrocellulose filter, and hybridization to $^{32}$P-M-MuLV cDNA. c, Cytoplasmic; n, nuclear RNA. a) A photograph of the RNA after electrophoresis and staining with ethidium bromide. The visible bands in the nuclear RNA preparations are mouse ribosomal RNA which was added as carrier during preparation. b) An autoradiogram of the RNA blot after hybridization to M-MuLV cDNA. The first 2 lanes show different exposures of total RNA prepared from M-MuLV-producing fibroblasts analyzed in parallel.

necessarily indicative of the presence of transcripts of this size. They merely show that a substantial portion of the transcripts is representative for the entire viral genome. We believe that the ~8.8 kb band observed after hybridization of the RNA to PvuII fragments (Fig. 4, lanes c) is the result of inefficient hybridization of a 72 bp PvuII fragment produced by cleavage at two closely adjacent PvuII sites within the LTR sequences (Fig. 1).

Northern blot analyses of the RNA from injected oocytes showed no discrete bands of virus-specific RNA. In M-MuLV-infected fibroblasts two virus-specific RNA species of 8.8 and 3.0 kb are detectable (Fig. 5b, lane 1). In contrast, in the oocytes injected with Mov-7 DNA (Fig. 5b, lane 3) or Mov-3 DNA (Fig. 5b, lane 4) most of the virus-specific transcripts were of heterogenous length...
Fig. 6: S1-mapping of 5' termini of M-MuLV-specific RNA. RNA from 2 different lines of M-MuLV-producing fibroblasts (a,c), M-MuLV virions (b), uninjected oocytes (d), and oocytes injected with Mov-7 DNA (e), pMov-3 DNA (f) and Mov-3 DNA (g) were analyzed by S1-mapping using a 5' end-labeled 515 bp Sau 3a fragment (compare Fig. 1). Ten μg each of cellular and oocyte RNA, approx. 20 ng of 70S RNA and 250 ng of the DNA probe were used.

ranging in size from M-MuLV genome length to less than 0.5 kb. This was not due to a degradation of the RNA during preparation, as judged by ethidium bromide staining of the gel (Fig. 5a). In pMov-3 DNA-injected oocytes (i.e., in the presence of pBR322 sequences) most of the transcription products were smaller than 2 kb (Fig. 5b, lane 2).

Correct Initiation of Transcription at the Viral Promoter in the 5' LTR

The heterogeneity and relatively small size of the M-MuLV-specific transcripts made in the oocytes could have been the result of incorrect initiation of transcription or of a premature termination and/or rapid degradation of the RNA synthesized. We therefore determined the 5' termini of the synthesized RNA by S1 nuclease mapping and comparison to authentic M-MuLV genomic RNA and mRNA from virus-producing cells. The probes used for these experiments are illustrated in Fig. 1. Hybridization of RNA initiated at the cap site in the 5' LTR to the 5' end-labeled 515 bp
Sau 3a fragment should produce a hybrid of 161 bp (Fig. 1, ref. 33). Fig. 6 shows that we were able to detect such a band after hybridization of the probe to M-MuLV genomic RNA and RNA from M-MuLV-producing fibroblasts as well as to RNA from oocytes injected with Mov-3 or Mov-7 DNA. In addition to this main band, there was a series of minor bands, the pattern of which was almost identical with the RNA from injected oocytes and authentic M-MuLV RNAs. We conclude from these results that the oocytes correctly use the viral promoter located in the 5' LTR to initiate transcription of viral RNA when injected with Mov-3 or Mov-7 DNA. Moreover, the Sl-mapping was done in 25- to 50-fold probe excess. Therefore, the intensity of the 161 bp band in DNA-injected oocytes in comparison to the band detected in M-MuLV-producing fibroblasts indicates that most or all of the virus-specific transcripts made in the oocytes are initiated correctly. In contrast, with RNA from oocytes injected with pMov-3 DNA, i.e., containing pBR322 sequences, the main band protected had the size of the complete probe (Fig. 6, lane f). This could be indicative of a readthrough from a pBR322 promoter into viral sequences. Alternatively, this band could be produced by RNA sequences transcribed from the 3' LTR which are expected to hybridize to regions of the probe upstream of the cap site. This possibility is supported by the fact that also M-MuLV genome RNA was able to protect a band with the size of the complete probe (Fig. 6b). A longer exposure of the autoradiogram, as well as the results of additional experiments, revealed that also in pMov-3 DNA-injected oocytes a substantial portion of the virus-specific transcripts was initiated at the correct site (data not shown). In addition to the bands at 161 and 515 bp, we found in the Sl-mapping experiments very reproducibly bands with a size of 190, 220 and 400 bp (Figs. 6 and 7). The origin of these bands has not been further studied.

No Detectable Transcription of Downstream Cellular Sequences from the 3' Viral Promoter

Since the coding sequences of retroviruses are flanked by LTR sequences (13, compare Fig. 1) and an additional promoter is located at the 3' end of the proviral genome, there is the possibility of downstream promotion from the 3' LTR. We have tested this possibility by probing the RNA synthesized in the injected
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Fig. 7: Analysis of initiation at the 3' promoter by S1-mapping. RNAs from uninjected or Mov-7 DNA-injected oocytes were analyzed by S1-mapping using a 5' end-labeled 515 bp Sau 3a fragment from the 5' LTR or a 800 bp PvuII fragment containing sequences from the 3' LTR and flanking Mov-7 mouse DNA (compare Fig. 1). Lane a: 515 bp Sau 3a fragment; lane b: 515 bp Sau 3a fragment digested with KpnI; lanes c and d: RNA from uninjected and Mov-7 DNA-injected oocytes, respectively, mapped with the 515 bp Sau 3a fragment; lane e: 800 bp PvuII fragment; lane f: 800 bp PvuII fragment digested with KpnI; lanes g and h: the same RNA from uninjected and Mov-7 DNA-injected oocytes, respectively, mapped with the 800 bp PvuII fragment.

Most of the Virus-Specific Transcripts are Confined to the Nucleus

The finding that most of the virus-specific transcripts after oocytes with probes complementary to the 3' LTR and flanking cellular sequences of Mov-3 and Mov-7 DNA (see Fig. 1). The result of such an experiment with RNA from Mov-7 DNA-injected oocytes is shown in Fig. 7. Hybridization of this RNA to the 515 bp Sau 3a fragment produced the expected 161 bp hybrid (Fig. 7d). In contrast, when the same RNA was hybridized to the 800 bp PvuII fragment, no hybrids were detectable (Fig. 7h). This suggests that the viral promoter located in the 3' LTR is used with a much lower (50- to 100-fold) frequency, if at all, for initiation of RNA synthesis as compared to the 5' promoter, although the surrounding sequences are identical.

Fig. 7: Analysis of initiation at the 3' promoter by S1-mapping. RNAs from uninjected or Mov-7 DNA-injected oocytes were analyzed by S1-mapping using a 5' end-labeled 515 bp Sau 3a fragment from the 5' LTR or a 800 bp PvuII fragment containing sequences from the 3' LTR and flanking Mov-7 mouse DNA (compare Fig. 1). Lane a: 515 bp Sau 3a fragment; lane b: 515 bp Sau 3a fragment digested with KpnI; lanes c and d: RNA from uninjected and Mov-7 DNA-injected oocytes, respectively, mapped with the 515 bp Sau 3a fragment; lane e: 800 bp PvuII fragment; lane f: 800 bp PvuII fragment digested with KpnI; lanes g and h: the same RNA from uninjected and Mov-7 DNA-injected oocytes, respectively, mapped with the 800 bp PvuII fragment.

Most of the Virus-Specific Transcripts are Confined to the Nucleus

The finding that most of the virus-specific transcripts after
injection of M-MuLV DNA were small and, in contrast to injected mRNA, not efficiently translated into virus-specific proteins (compare above) prompted us to determine the distribution of virus-specific RNA between the cytoplasmic and nuclear compartments after injection of M-MuLV DNA. Fig. 5 shows that most (>90%) of the virus-specific RNA detectable by Northern blot analysis was confined to the nucleus of the injected oocytes.

DISCUSSION

We have shown in this paper that different clones of genomic mouse DNA containing a M-MuLV proviral copy flanked by different cellular sequences are being transcribed into virus-specific RNA when injected into the nucleus of X. laevis oocytes. Initially, we attempted to use the oocyte transcription system to identify flanking mouse cellular sequences exerting a regulatory function upon the expression of the proviral genome, e.g., in Mov-7 or Mov-10 mice that do not express infectious virus in vivo. However, there was no correlation between the expression of infectious virus in the different Mov-substrains and the transcription efficiencies of the respective cloned proviral DNA in the oocytes. Rather, Mov-7 DNA, the proviral genome from the non-viremic Mov-7 mouse substrate, was reproducibly transcribed with a 2- to 5-fold higher efficiency than the proviral DNA from the viremic Mov-3 mouse strain. Moreover, the flanking cellular sequences of Mov-7 DNA had no significant influence upon transcription of the proviral genome (Table I). This is in agreement with recent results which show that in Mov-7 and Mov-10 mice the failure to induce infectious virus is due to mutations in the gag-pol region of the proviral genomes (34).

The accumulation of virus-specific RNA in the oocytes after injection of cloned M-MuLV DNA is rather low: We find between 0.2 and 0.6 ng RNA per ng DNA injected after 24-48 hrs (Table I). Similar values have been reported by others (7,8,30). Several factors could possibly account for these low transcription efficiencies: (i) A limited supply of polymerase II or other essential factors; this seems unlikely, since we have injected between 5 and 10 ng DNA per oocyte, an amount for which the transcriptional capacity of an oocyte is sufficient (8). (ii) Only a
small fraction of the injected DNA molecules are being transcribed. Trendelenburg et al. (35) have made an observation supporting this view: After injection of a cloned chicken ovalbumin gene most of the injected gene copies could be visualized in electron micrographs as apparently inactive chromatin and only a minority was found in active transcriptional complexes. (iii) The virus-specific RNA transcribed in the oocyte is rapidly being degraded. Our data, especially the rather small size of the virus-specific transcripts, support this possibility. The final level of hybridization in the Crt analyses was usually about 80% (Fig. 2). This and the hybridization analysis of $^{32}$P-labeled RNA (Fig. 3) show that the virus-specific RNA made by the oocytes was complementary to the entire viral genome and that premature termination was not a main reason for the small size of the RNA. Similarly, the results of the Sl-mapping experiments (Fig. 6) argue against a significant degree of unspecific initiation of transcription within the coding sequences of the proviral genome. Equal amounts of RNA from virus-producing fibroblasts and DNA-injected oocytes resulted in protected bands of approximately equal intensity.

The small size of the virus-specific RNA and its confinement to the nucleus (Fig. 5) appeared to be a reason for our inability to detect virus-specific proteins after injection of proviral DNA, whereas after injection of a comparable amount of mRNA viral protein was readily detectable (Fig. 3). Similar low translational efficiencies have been described by others. After injection of SV40 DNA (28) and chick ovalbumin DNA (30) only a small fraction (<1%) of the transcribed RNA was translated into protein. The reason for this is not clear; however, the picture is reminiscent of the situation in other eukaryotic cells, where most of the RNA transcribed never leaves the nucleus but is turned over rapidly. On the other hand, it is conceivable that certain structural features of the cell, such as the nuclear matrix, play a regulatory role by being a prerequisite for a proper transcription, processing, and transport of functional mRNA molecules. These requirements are certainly not met when large quantities of foreign genes are injected into and transcribed by heterologous cells, and a rapid turnover of the
transcripts from such genes may well be the result.

Activation of cellular oncogenes by "downstream promotion" or "promoter insertion" is thought to be an important mechanism of tumorigenesis and has, for example, been shown to be involved in the induction of lymphomas by avian leukosis virus (37,38). Our attempts to detect promotion of transcription of downstream cellular sequences from the promoter in the 3' LTR were not successful (Fig. 7). This shows that the 3' viral promoter was used in the oocyte with a much lower frequency, if at all, for initiation of RNA synthesis than the 5' promoter. It has been shown that 3' LTR sequences from avian as well as from murine retroviruses can function as promoter in cell-free transcription systems (10,11) or when linked to a heterologous gene and transfected into cells (39). In the in vitro transcription system initiation of transcription occurs within the 5' and 3' LTR with equal efficiency (11). Therefore, the reason for the difference in promoter activity of the 5' and 3' LTR of M-MuLV that we find remains unclear.

It is possible that differences in the chromatin conformation of the 5' and 3' LTR sequences of the integrated proviral genome result in a preferential initiation at the 5' promoter and/or an efficient termination of all viral transcripts at the 3' poly(A) site resulting in a preferred synthesis of genome size viral RNA. This may inhibit initiation at the 3' promoter sequence. These regulatory mechanisms, which would be expected to be, at least in part, active in the oocyte system, might not be recognized by the in vitro transcription system or when a small restriction fragment is used as template of transcription.

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