Rous sarcoma virus (RSV) is an oncogenic avian retrovirus that undergoes nonpermissive infections in mammalian cells. When RSV is introduced into mammalian cells, either by infection with certain strains of RSV (1, 10, 33-35) or by transfection of viral DNA (7, 26), occasional clones arise that show a transformed phenotype. In these cell lines, integrated copies of proviral DNA are present in the host chromosomes (34, 35), and a low level of unprocessed gag precursor Pr76 is produced (11, 36, 37). However, neither infectious nor noninfectious particles are produced (21, 32). However, infectious virus can be rescued from RSV-transformed mammalian cell lines by fusion with permissive chicken embryo fibroblasts (CEF) (5, 15, 24, 31, 32). The levels of viral RNA in RSV-transformed mammalian cell lines are 1 to 3 orders of magnitude lower than those in infected CEF (3, 6, 19). The predominant viral RNA species in these transformed mammalian cell lines is spliced src mRNA; only small amounts of unspliced and env mRNA are present as well as minor amounts of several other unidentified RNAs (8, 9, 19).

It is not clear to what extent selection for rare transformants in a population of infected cells might affect the types and quantitative distributions of mRNAs in RSV-transformed cells. To avoid this selective bias, we examined viral RNA from NIH 3T3 cells transfected with proviral DNA clones of the Prague A strain of RSV. Also, a comparison of RNA processing of transcripts from a molecularly cloned RSV genome in cells derived from different species should provide information about the maintenance of balanced splicing in different types of infected cells (for a review, see reference 25).

In this report we show that there is a marked increase in the efficiency of splicing of the viral RNAs in NIH 3T3 cells relative to CEF and that NIH 3T3 cells efficiently use a cryptic 3' splice site (5'ss) within the env coding sequence which is silent in CEF. The activation of the env 5'ss in NIH 3T3 cells leads to further processing, resulting in the production of two different forms of the src mRNA. This may have implications for the expression of src in mammalian cells. It may also account in part for the lack of permissiveness in mouse cells for virus replication.

MATERIALS AND METHODS

Plasmids. pJD100, an infectious nonpermuted proviral clone of a Prague A strain of RSV contained in pBR322, was obtained as a gift from J. Thomas Parsons, Department of Microbiology, University of Virginia, Charlottesville. The RNase mapping templates pMap47 and pMap*10 (Fig. 1) were constructed and characterized (Berberich and Stoltzfus, unpublished data). Plasmid pMPM13, a mutant at the src 3' splice site (3'ss), was constructed in the following manner. A subclone of pJD100 that contained the sequence from the ScaI site at nucleotide (nt) 6865 to the TthIII-I site at 7330 was cleaved with PstI (single site at nucleotide 7048), treated with T4 DNA polymerase in the presence of the four deoxyribonucleotides to remove the 3' extensions, and blunt end ligated. The ScaI-NaeI fragment (nucleotides 6865 to 7169) was then inserted into pJD100 to create pMPM13. During this cloning step, a small deletion in the 3'sc exon was also created that altered the protected RNA fragments in the RNase mapping experiments (see Results).

Cell culture and DNA transfections. Secondary CEF isolated from embryonated eggs that were negative for both group-specific antigen and chicken-helper factor (SPAFAS,
Inc., Gaithersburg, Md.) supplemented with 10% (vol/vol) tryptose phosphate broth and 5% (vol/vol) calf serum (SGM). Mouse NIH 3T3 cells were cultured in Dulbecco minimum Eagle medium containing 10% (vol/vol) calf serum. CEF were transfected by using a DEAE-dextran procedure described previously (22). Purified plasmid DNA (15 μg) in 6 ml of serum-free SGM made to a final concentration of 0.05 M Tris (pH 7.4) was used to transfect subconfluent 100-mm plates of CEF in the presence of 200 μg of DEAE-dextran per ml. After 30 min at room temperature, 2 ml of the transfection mix was removed, and 10 ml of serum-free SGM was added. The plates were then incubated for 3 h at 37°C, washed with 3 ml of SGM, fed with 10 ml of SGM, and incubated at 37°C. The medium was changed 15 to 20 h later with 10 ml of SGM. NIH 3T3 cells were transfected by calcium phosphate precipitation (13). Plasmid DNA (25 μg) was used for each 150-mm plate. The DNA precipitate was left on the cells for 4 h, and the cells were treated with 1× HEPES-buffered saline containing 20% (vol/vol) glycerol for 4 min as described previously (33).

RNA isolation and analysis. Whole-cell RNA at 48 h after transfection was isolated by the guanidine hydrochloride method essentially as described by Strohman et al. (29). Northern RNA blot analysis with SP6 riboprobes (17) was carried out on formaldehyde-agarose gels (16). The RNase protection mapping was carried out essentially as described previously (28), except that the hybridizations were carried out at 57°C for 14 h and the RNA digestion were done at room temperature for 30 min with 6 μg of RNase A per ml and 300 μg of RNase T1 per ml. The protected fragments were denatured by heating at 85°C for 10 min and separated on a 7 M urea denaturing 6% polyacrylamide gel at 500 V for 3 to 5 h.

Materials. [α-32P]UTP (800 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. Restriction enzymes, RNase A, RNase T1, T4 polymerase, T4 ligase, and proteinase K were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. SP6 polymerase was obtained from Promega Biotec Co., Madison, Wis. Plasmid pSP18 was obtained from Bethesda Research Laboratories, Inc., Bethesda, Md.

RESULTS

Characterization of viral mRNAs in transfected NIH 3T3 cells. We first compared the viral RNA produced in CEF and NIH 3T3 cells after transfection of the wild-type Prague A RSV clone, pJD100. Two different tandem radiolabeled riboprobes complementary to sequences spanning each of the major splice sites were used (Fig. 1). After hybridization with these tandem probes and RNase digestion of the resulting hybrids, each complementary probe region (5′ region, env, and src) produced protected fragments of unique size from unspliced RNA and different unique-sized fragments for spliced mRNAs, which were separated by electrophoresis on a polyacrylamide gel. RNase protection results for whole-cell RNA harvested from CEF and NIH 3T3 cells at 48 h after transfection are given in Fig. 2. The origin of the various fragments produced by RNase protection mapping and their sizes in nucleotides are also diagrammed (Fig. 2). In the first set of experiments, the 32P-labeled probe was synthesized from the pMap#10 template (Fig. 2, lanes 5 to 8). No qualitative differences were seen in the mobilities of the protected fragments derived from the 5′ region (375 nt unspliced, 143 nt spliced) and the src 3′ ss region (347 nt unspliced, 276 nt spliced). However, the 180-nt fragment protected by the normal spliced env mRNA in CEF cells was absent in the NIH 3T3 lane (Fig. 2, compare lanes 6 and 8). Instead, two other protected env
fragments were present whose sizes were approximately 230 and 160 nt. To more clearly resolve the 276-nt spliced src and 263-nt unspliced env fragments, another probe synthesized from pMap47 (Fig. 1) was used. Note that pMap47 was derived from pMap#10 by deleting 47 nt upstream of the env 3'ss, i.e., within the env intron. As discussed above with the pMap#10 probe, no qualitative differences in the mobilities of the src or 5' bands were seen with the 3T3 and CEF fragments were compared (Fig. 2, compare lanes 2 and 4). However, the 230-nt env band was no longer present. Instead, a band migrating slightly slower than the 180-nt normal env spliced band was present (Fig. 2, lane 4). The 160-nt env band, however, did not change. The results in Fig. 2 implied that normally spliced env mRNA was not detectable in transfected NIH 3T3 cells. The presence of protected fragments not present in CEF suggested that the processing of env mRNA might be different in NIH 3T3 cells.

It has been shown in several laboratories that a cryptic 5' G/GUAAGA (at nt 5237) just downstream from the normal env 3'ss (at nt 5078) is activated in avian cells under some circumstances (12, 18, 23). If this cryptic splice site was used to further splice the env mRNA in NIH 3T3 cells to form a double-spliced RNA, it would be expected to protect a 159-nt fragment (the distance between the env 3'ss at base 5078 and the env cryptic 5'ss at nt 5237) with both pMap47 and pMap#10 probes. This size agrees well with the ~160-nt env-specific bands seen in the experiments in Fig. 2 (lanes 4 and 8). A single-spliced RNA in which the cryptic 5'ss is used to splice to a downstream 3'ss would be expected (Fig. 1) to protect a 186-nt fragment with the Map47 probe and a 233-nt fragment with the Map#10 probe. The evidence shown in Fig. 2 is consistent with this hypothesis. Note that the 186-nt fragment was not resolved from the 180-nt spliced env fragment (Fig. 2, lane 4). However, the absence of the 180-nt band and the appearance of the 233-nt band in the Map#10 probe products (Fig. 2, lane 8) suggests that these bands arise as products of the single-spliced RNA.

We suspected that the most likely splice site to be used as an acceptor for the cryptic 5'ss in env would be the src 3'ss. As a test to determine whether the src 3'ss was used in the cryptic splicing, we constructed a mutant of pJD100, pPM13, in which the src 3'ss was destroyed by a 4-base-pair deletion. The results of RNase mapping with the Map47 probe are given in Fig. 3. Because of a discontinuity due to a second deletion arising downstream of the src 3'ss, cleavage of the probe occurred at this site when the hybrid RNAs were digested with nuclease. Cleavage of the probe also occurred at the discontinuity created by the mutation created at the src 3'ss (see Materials and Methods). This resulted in the production of three protected src probe fragments arising from unspliced RNA. The positions of two of these fragments (148 and 130 nt) are indicated in Fig. 3, a third, smaller fragment (71 nt) is not shown. In addition, a minor band at the position of the normal spliced src probe (276 nt) resulted from incomplete RNase digestion. In addition to these bands arising from unspliced src, the normal expected 5'-specific protected bands (375 and 143 nt), and the unspliced env band (216 nt), a band migrating at the normal spliced env position at 180 nt was present. The mobility of this fragment was not altered when the RNA was analyzed by using a riboprobe generated from pMap#10 rather than pMap47 (data not shown). Thus, when the src 3'ss was deleted, normal single-spliced env mRNA was formed. The band at approximately 160 nt (arrow in Fig. 3), which was diagnostic for splicing from the cryptic 5'ss, was
not detectable. These results indicate that formation of the double-spliced mRNA from the env cryptic 5′ss requires the src 3′ss.

Evidence for the presence of double-spliced RNA species in NIH 3T3 cells was also obtained by Northern blot analysis. A 32P-labeled riboprobe specific for the env splice site region was prepared and used to probe the Northern blots (Fig. 4). A band at ~4.0 kilobases (kb) was present in all lanes, including the negative control lanes; this band corresponded to the position of 28S rRNA as determined by ethidium bromide staining. Attempts to remove the nonspecific binding of this probe by RNase digestion and further washing of the blot resulted in the loss of the virus-specific signals as well. We do not understand the reason for the affinity to 28S rRNA. However, since the position of the 28S rRNA did not overlap the positions of the specific viral mRNAs, the artifact band did not interfere with the analysis of the viral RNAs. RNA from wild-type pJD100-transfected cells hybridized to the env-specific probe is shown in Fig. 4, lane 1. Two specific bands were detected, a 9.3-kb band corresponding to the full-length unspliced RNA and a 2.8-kb band corresponding to the double-spliced mRNA, which is slightly larger than normal src mRNA. A specific band was

FIG. 3. Effects of inactivation of the src 3′ss on env splice site usage in NIH 3T3 cells. (A) Schematic representation of the plasmids used in this experiment. (B) RNase protection map with a probe from pMap47 and RNAs isolated from NIH 3T3 cells transfected with pMPM13 (lane 1), pJD100 (lane 2), and no DNA (lane 3). The identities and locations of the various protected fragments are indicated. Labeling of bands is as described in the legend to Fig. 2. The asterisk (+) indicates a background band found in the control RNA. The two smaller src-specific bands arising from unspliced (un)RNA in pMPM13 transfected cells are also indicated and migrate faster and slower than the 143-nt spliced 5′ band. The position of the 160-nt fragment diagnostic for the double-spliced RNA is indicated by an arrow.

FIG. 4. Northern blot analysis of mouse NIH 3T3 cells transfected with cloned DNA. Whole-cell RNA was isolated 48 h after transfection and fractionated on an 1.2% agarose–formaldehyde gel. RNA isolated from NIH 3T3 cells (lane 1) and CEF (lane 3) transfected with pJD100 or the same cells with no DNA (lanes 2 and 4) were electrophoresed and probed with a 32P-labeled riboprobe specific to the env splice site (the same region protected by the env portion of pMap47). The amounts of RNA applied to the gel were 30 μg in lanes 1 and 2 and 10 μg in lanes 3 and 4.
not visible at the position of normal env mRNA (4.6 kb) which migrated slower than the ∼4.6-kb RNA background band (Fig. 4). The results of a Northern blot of RNAs from pJD100 (lane 3) and mock-transfected CEF (lane 4) are also given in Fig. 4. Specific bands of 9.3 and 4.6 kb were present, corresponding to the full-length unspliced and normal single-spliced env RNAs, respectively. Confirming the results of the RNase protection assays (Fig. 2), a specific double-spliced env-containing RNA band at the position of the src mRNA (2.6 kb) was not detected in transfected CEF.

Thus, our results suggest that RSV RNA is processed in NIH 3T3 cells (Fig. 5). A minor (cryptic) 5'ss is located in the 5' region of the env gene, probably at the consensus sequence beginning at nt 5237 since this is the only potential consensus splice donor site in this region of the RSV genome. Use of this cryptic 5'ss and the src 3'ss in NIH 3T3 cells resulted in the formation of both a double-spliced mRNA and a single-spliced mRNA.

Comparison of amounts of spliced and unspliced viral RNAs in transfected CEF and NIH 3T3 cells. It was obvious from the results given in Fig. 2 that there were significant quantitative differences between NIH 3T3 cells and CEF in the relative amounts of the spliced products. The overall level of splicing, as determined by fragments derived from the 5' region of the tandem riboprobe (for both pMap47 and Map±10, the 375- and 143-nt fragments), is clearly increased in NIH 3T3 cells. Most of the increased splicing occurred at the src 3'ss, as can be seen by comparing the relative amounts of the 276-nt spliced src fragments in CEF and NIH 3T3 cells (Fig. 2, compare lanes 2 and 4 and lanes 6 and 8). The autoradiograms from a number of separate experiments were scanned, and the relative molar amounts of spliced and unspliced RNA transcripts were determined (Table 1). In the case of CEF, the overall extent of viral RNA splicing was determined to be approximately 25%. In NIH 3T3 cells, on the other hand, the extent of splicing was considerably higher, approximately 60 to 70%. The double-spliced RNA comprised about 7%, and the RNA that was singly spliced from the cryptic 5'ss to the src 3'ss comprised about 3%, of the total viral RNA in NIH 3T3 cells transfected with pJD100. From these values, the amount of normal single-spliced src mRNA was calculated to be approximately 50 to 60% of the total viral RNA, i.e., the difference between the number of RNA molecules spliced at the src 3'ss and the number of RNA molecules resulting from the cryptic splicing in env. It is of interest that the fraction of total viral RNA spliced at the env 3'ss was not significantly different in NIH 3T3 cells and CEF (11 versus 13%). However, the fraction of single- and double-spliced RNA spliced at the src 3'ss was increased approximately fivefold in NIH 3T3 cells (63 versus 13%).

It appeared from the Northern blot data (Fig. 4, lane 1) that the env probe hybridized most strongly to the 2.8-kb RNA than to the full-length 9.3-kb RNA. This is clearly inconsistent with the RNase protection data given in Table 1. We believe that this discrepancy arises because of less

<table>
<thead>
<tr>
<th>RNA</th>
<th>% CEF ± SD</th>
<th>% NIH 3T3 cells ± SD</th>
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<tbody>
<tr>
<td>Unspliced</td>
<td>74.1 ± 4.0</td>
<td>37.3 ± 4.7</td>
</tr>
<tr>
<td>Single-spliced src</td>
<td>13.0 ± 2.6</td>
<td>51.6 ± 4.3</td>
</tr>
<tr>
<td>Single-spliced env</td>
<td>12.8 ± 2.3</td>
<td>ND</td>
</tr>
<tr>
<td>Double-spliced env</td>
<td>ND</td>
<td>7.6 ± 2.0</td>
</tr>
<tr>
<td>Cryptic single-spliced env</td>
<td>ND</td>
<td>3.6 ± 1.2</td>
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* For CEF RNA, the integrated values obtained by densitometric measurements for the unspliced env, spliced env, and spliced src bands were determined (Fig. 1). For NIH 3T3 RNA, the integrated values for the cryptic single and double-spliced bands were also determined. The integrated values were normalized for the appropriate base compositions of the bands, and the molar ratios of each RNA species were determined. The percentage of the total viral RNA for each RNA was then calculated. For the NIH 3T3 cells, the contribution of the cryptic spliced env species (double and single spliced) was subtracted from the src band value. Standard deviations are based on 10 determinations for CEF and 7 determinations for NIH 3T3 cell RNA.

* ND, Not detected.
efficient transfer of higher-molecular-weight RNA species in the Northern blot procedure. In fact, we have noted considerable differences in the relative intensities of the two bands in different Northern blot experiments (data not shown). Thus, our data indicate that the RNase protection assay is the more appropriate method to obtain accurate quantitative data of viral RNA levels.

It was difficult to directly compare the total levels of RNA transcripts in CEF and NIH 3T3 cells since the amount of DNA transfected, the transfection protocol, and the exposure times of the autoradiograms were different. However, based on the data of Fig. 1 as well as other experiments, we estimated that the level of viral RNA in transfected cells was 10- to 20-fold lower in NIH 3T3 cells than in CEF. This is in agreement with other studies showing reduced levels of viral RNA in RSV-transformed mammalian cell lines (3, 6, 19).

Comparison of stabilities of RSV RNA in NIH 3T3 cells and CEF. It was possible that the relatively larger amount of spliced RNA compared with the amount of unspliced RNA in NIH 3T3 cells (Table 1) might be due to the instability of the unspliced RNA in NIH 3T3 cells versus CEF rather than to the differences in RNA splicing. Indeed, the lability of unspliced RNA transcribed from certain RSV gag mutants is responsible for reduced ratios of unspliced to spliced RNA in CEF (2). To measure RNA stabilities, NIH 3T3 cells were transfected for 48 h with wild-type pJD100 and then treated with actinomycin (1 μg/ml) to inhibit further transcription. Whole-cell RNA was then harvested at various times (Fig. 6). It was indicated in previously reported results from this laboratory that in CEF the env mRNA is most stable (half-life, ~15 h), the src mRNA is moderately stable (half-life, ~10 h), and the genomic unspliced message is the most labile (half life, ~8 h) (27). In the experiment shown in Fig. 6 the src mRNA appeared to be the least stable (half-life, ~4 h) in NIH 3T3 cells; the unspliced message was slightly more stable (half-life, ~4.5 h). As shown above, we were unable to detect any normal env mRNA in NIH 3T3 cells. The two mRNAs resulting from the use of the cryptic splice site appeared to have stabilities greater than that of the other viral RNAs. Overall, the RSV RNA was less stable in NIH 3T3 cells than in CEF. From these data we conclude that the observed steady-state ratios of spliced to unspliced RNA in NIH 3T3 cells are likely to underestimate the actual extent of the splicing because the unspliced RNA is more stable than the major spliced species, the src mRNA.

DISCUSSION

We have obtained evidence for altered splicing of RSV RNA in mouse NIH 3T3 fibroblasts compared with RSV RNA in CEF. We have demonstrated a significant quantitative shift in the distribution of viral mRNAs toward shorter spliced mRNAs 48 h after transfection. RSV RNA has been shown to be mostly spliced in RSV-transformed mammalian cell lines (8, 9, 19). Experiments from this laboratory have indicated that the types of mRNA and the extent of RNA splicing are identical in CEF transiently transfected by cloned viral RNA and in CEF synchronously infected by virus at a high multiplicity of infection (Berberich and Stoltzfus, unpublished data). Therefore, RNA processing of viral RNA in transfected cells appears to accurately reflect events in virus-infected cells. Our results suggest that the preponderance of spliced mRNA in mammalian cells does not reflect genetic selection for an overspliced phenotype in rare transformed cells, since this also occurs in transient expression assays without selection for transformation. The extent of splicing may be even higher than that indicated by the steady-state ratios of spliced to unspliced RNA, since the src mRNA, which is the major spliced species in NIH 3T3 cells (Table 1), appears to be less stable than the unspliced RNA in NIH 3T3 cells.

Our further results indicate that qualitative differences exist in permissive CEF versus nonpermissive mammalian cells. We have shown that normal env mRNA is not detectable in the RSV DNA-transfected NIH 3T3 cells. Instead, we have obtained evidence for two novel spliced RNA species, a 2.8-kb double-spliced mRNA derived by further splicing of env mRNA and smaller amounts of a 7.5-kb single-spliced RNA (Fig. 5). Processing of both of these RNAs utilizes a cryptic 5'ss, which appears to be at the splice consensus sequence beginning at nt 5237 within the env coding sequence. This 5'ss has been used previously (12, 18, 23). The transformation-defective RSV deletion mutant td109 is believed to acquire c-src by splicing from the cryptic 5'ss in env to the first exon of c-src in infected chickens (23). Fung et al. (12) found that the env cryptic 5'ss was involved in the induction of erythroblastosis by proviral insertion of avian leukosis virus within the cellular c-erbb locus. RNA transcripts initiated at the avian leukosis virus 5'ss' long terminal repeat were spliced from the cryptic 5'ss in env to a 3'ss within c-erbb. Finally, a recombinant RSV-based retroviral vector carrying the c-erbb cDNA produced two c-erbb mRNAs. One of these mRNAs is directly spliced from the major 5'ss in gag to the c-erbb 3'ss; the other is double spliced from the gag 5'ss to the env 3'ss and further spliced from the env cryptic 5'ss to the c-erbb 3'ss (18). Thus, although we did not detect spliced RNAs by using the
cryptic 5'ss in CEF, it is clear that under some circumstances and in some tissues the cryptic splice site can be used in chicken cells, and our results cannot exclude the presence of minor amounts of such RNA. The cryptic splice site in env could be used to create novel mRNAs in which the env signal peptide sequence is fused to downstream open reading frames by using cryptic 3'ss within the env and src genes. Further studies with more sensitive techniques are required to obtain evidence for such putative mRNAs.

It is possible that the presence of double-spliced mRNA in NIH 3T3 cells may affect src gene expression. The 5' leader of the normal src mRNA contains four short upstream open reading frames; the 5' distal upstream open reading frame is initiated by the gag/env AUG (4, 30). However, the double-spliced mRNA appears to be identical to normal src mRNA, except for an in-frame insertion of the leader portion of the env mRNA, which includes the signal peptide sequence. If src can, indeed, be translated from the double-spliced mRNA by reinitiation, translation of the upstream orf may have effects on downstream src expression. For instance, RSV env mRNA is known to be associated with membrane-bound polysomes, whereas src mRNA is associated with cytoplasmic polysomes (14). If the double-spliced mRNA were distributed like the env mRNA, it could conceivably affect the cellular targeting of pp60src synthesized by the two types of mRNA. Further work however must be done to fully characterize the double-spliced mRNA.

The absence of normal env mRNA shown above in transduced NIH 3T3 cells may explain in part the nonpermissiveness of mouse cells to infection by RSV. However, this is clearly not the only defect preventing virus replication. Wills et al. (38) have recently shown that the RSV gag precursor is not transported to the cytoplasmic membrane of mammalian cells and that this defect can be corrected by altering the N terminus to allow N-terminal myristylation. This mutation, however, when inserted back into an infectious RSV plasmid and transduced into mammalian cells, did not result in productive virus infection (J. Wills, personal communication). The level of transcription from the RSV long terminal repeat, as we have confirmed in this study in transiently transduced NIH 3T3 cells, has also been shown to be considerably lower in mammalian cells compared with the level in CEF (3, 6, 19). Therefore, defective mRNA splicing, lower proviral transcription, and inefficient protein processing may act synergistically to completely block virus replication in mammalian cells.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA28051 from the National Cancer Institute and by the Veterans Administration. S.L.B. was supported by Public Health Service National Research Service Award GM07091 from the National Institute of General Medical Sciences. L.P.T. was supported by a Career Award from the Veterans Administration.

We thank M. Stinson for critical reading of the manuscript and M. Reeve for typing.

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