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naf, a *trans*-Regulating Negative-Acting Factor Encoded within the Mouse Mammary Tumor Virus Open Reading Frame Region

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The mouse mammary tumor virus (MMTV) long terminal repeat (LTR) open reading frame (ORF) encodes a negative acting factor (*naf*). In our test system, *naf* mediates its effect in *trans* on another MMTV provirus in which the 5′ LTR has been replaced by that of Rous sarcoma virus. *naf* effects are evidenced at the level of transcriptional initiation rather than as reduced mRNA stability. The introduction of a premature termination codon into the MMTV LTR-encoded ORF abolishes the transcriptional down regulation localizing *naf* within the ORF. In addition, sequences in the *gag/pol* genes between +320 and +646 and between +3626 and +4590 relative to the site of transcription initiation are also involved in the MMTV-mediated transcriptional down regulation.

The genome of mouse mammary tumor virus (MMTV) contains an enigmatic open reading frame (ORF) located within the U3 region of the long terminal repeat (LTR) (7, 12). The biological functionality of this ORF is assumed since (i) the reading frame is highly conserved between different MMTV isolates (4, 17), (ii) a candidate ORF mRNA of 1.7 kb, containing mostly information from the LTR region, has been found in some infected cell types (21, 22), and (iii) in vitro translation of viral RNA corresponding to the 3′ end of the viral genome yields a series of methionine-rich proteins (5, 6, 19). However, the expression of the ORF protein has never been convincingly detected in vivo, possibly indicating that it is expressed at very low levels or at a critical, as yet unknown, point in the viral life cycle. The previous lack of cloned biologically active MMTV proviruses coupled with a suitable assay system has hindered the elucidation of the ORF function by mutation analysis.

Recently, we have reported the cloning of a biologically active MMTV provirus (16) (Fig. 1) and the subsequent construction of a two-component retroviral vector system based on this provirus. One of these components is a packaging cell line, RMC2h, which was designed to synthesize MMTV structural proteins in *trans* for MMTV-based vectors (18). This cell line contains an MMTV provirus which carries a Rous sarcoma virus (RSV) LTR in place of the MMTV LTR located at the 5′ end of the provirus (pRSV-MMTV) (Fig. 1). The presence of an RSV LTR ensures high-level expression of the MMTV structural proteins while hindering packaging and/or reverse transcription of the RNAs giving rise to these proteins (18). The second component is the vector construct (pWG29) (Fig. 1) which carries a selectable neomycin resistance gene under the transcriptional control of a thymidine kinase gene promoter at the expense of almost all of the *env*-coding sequences (9).

Transfection of the pWG29 vector construct into the RMC2h packaging cell line results in the production of recombinant virions with a titer of around 300 neomycin-resistant colonies per ml when assayed on the feline kidney cell line, CK (18). Although the titer of recombinant virus obtained by using this packaging cell line is almost 2 orders of magnitude greater than the titer obtained by using MMTV-producing helper cell lines (9, 18), it is still low when compared with titers obtained when retroviral vector systems based on murine leukemia retroviruses were used (8).

Retroviruses such as human immunodeficiency virus and bovine leukemia virus have been shown to encode regulatory factors that influence their expression (3, 11, 13, 14, 23). To determine whether the low titer of recombinant virus production from the MMTV-based retroviral system is due to an MMTV-encoded negatively regulating factor, we examined the effects of introducing the vector construct, pWG29, on the packaging construct, pRSV-MMTV, in the RMC2h cell line. The pWG29 vector carries a neomycin resistance gene allowing the selection of populations of transfected RMC2h cells. These cell populations were analyzed for the expression of MMTV proteins by Western immunoblotting. Antiserum directed against the major *gag* protein p28 recognizes the *gag* proteins p38 and p34 in a control population of pSV2neo-transfected RMC2h cells (Fig. 2, lane 1). p28 is not expected to be detected in the cells, since the cleavage of p34 to p28 occurs in mature virions (18). There is a slight, 1.5-fold induction in the amount of MMTV protein synthesized from the RSV promoter when the cells are cultured in the presence of dexamethasone (Fig. 2, lane 2) (Table 1). This is due to the presence of a weakly glucocorticoid-responsive element located within the RSV LTR (18). In contrast, the amount of *gag* proteins produced by a population of RMC2h cells on transfection of pWG29 (RMC2hpWG29) is considerably reduced (Fig. 2, lanes 3 and 4) (Table 1). There is no significant effect on glucocorticoid responsiveness (Table 1), demonstrating that this protein down regulation is independent of glucocorticoid. These results would suggest that the pWG29 construct is able, in *trans*, to negatively regulate expression from the pRSV-MMTV construct.

To determine whether negative regulation occurs at the level of transcription, correctly initiated RSV-MMTV transcripts were mapped by an S1 nuclease protection assay (1). A 2.0-kb *Pst*I fragment comprising the RSV LTR, a small region of the MMTV leader, and plasmid sequences was used to protect the RNA originating from the RSV LTR initiation site (Fig. 1). The hybridizing and thus nuclease-

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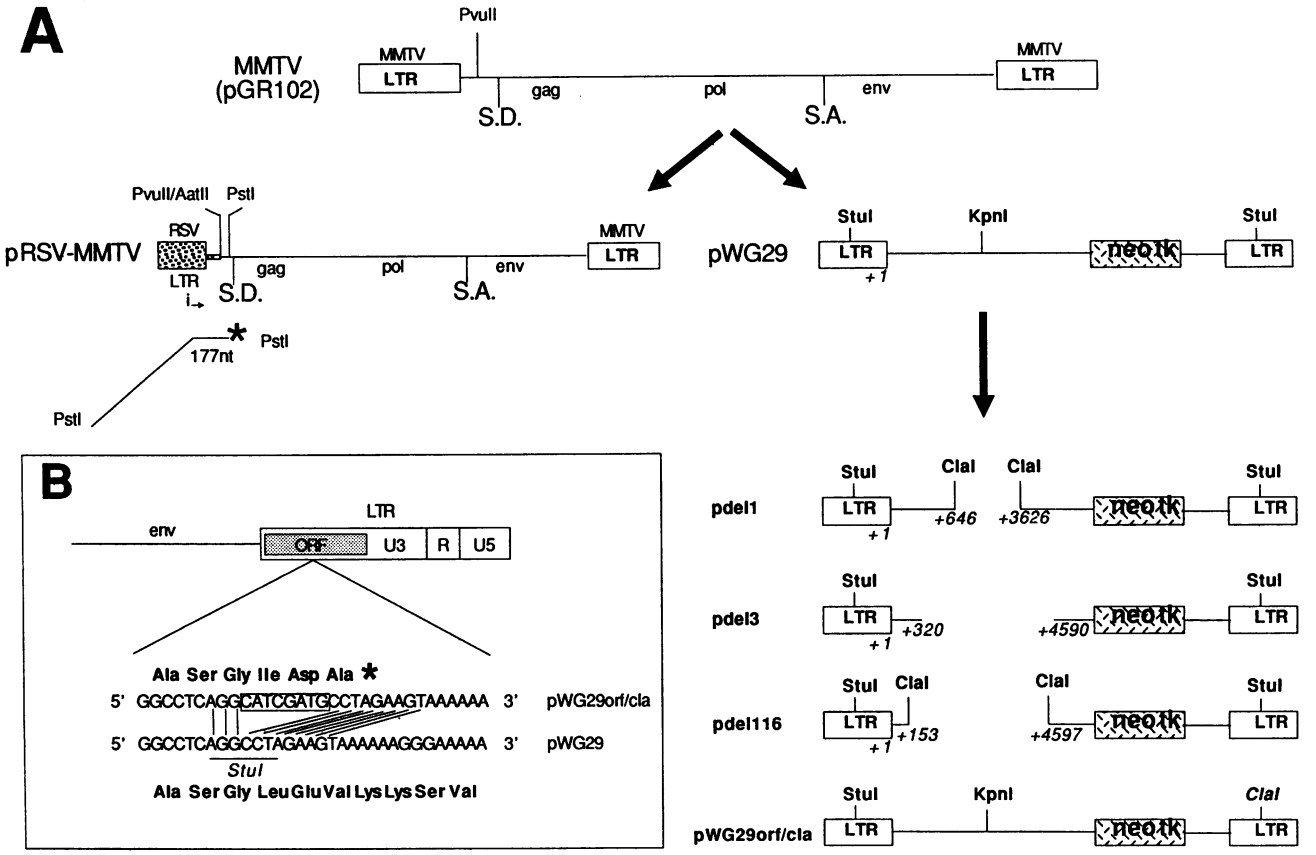

FIG. 1. (A) Schematic representation of MMTV constructs based on pGR102 (16). The plasmid pRSV-MMTV is the packaging construct carried by the RMC2h cell line (18). The 2.0-kb *PstI* end-labeled probe (*) used in the S1 mapping is indicated and contains 177 nucleotides complementary to the 5' end of chimeric RSV-MMTV RNAs. S.D., Splice donor; S.A., splice acceptor. The vector construct, pWG29, and derived constructs are also shown. The proviruses carry the herpes simplex virus thymidine kinase promoter (tk) linked to the neomycin resistance gene (neo) of Tn*5*. The transcriptional orientation of the thymidine kinase promoter is opposing that of the MMTV LTR. Indicated are the endpoints of the deletions relative to the transcription initiation site of MMTV. The plasmid pWG29orf/cla contains a *ClaI* linker inserted at the *StuI* site in pWG29, designed to frameshift ORF. (B) Frameshift verified by sequencing. The upper sequence is the verified nucleotide sequence of the pWG29orf/cla mutant provirus. Insertion of the *ClaI* linker (boxed) is expected to prematurely terminate (*) the ORF product, and the amino acid residues Leu-Glu-Val are replaced by Ile-Asp-Ala. Premature termination should result in the loss of about 10 kDa from the carboxy terminus of the ORF protein.

protected region should comprise 177 nucleotides, 46 of which originate from the RSV LTR and 131 of which derive from the MMTV leader (18). The predicted fragment of 177 nucleotides was protected by RNA from the RMC2h cell line (Fig. 3, lane 2), as was a second fragment of 178 nucleotides, presumably a minor site of transcription initiation.

When RNA from RMC2h cells transfected with pWG29 was tested in this experiment, there was a threefold decrease in the level of correctly initiated transcripts (Fig. 3, lane 3) (Table 1). Run-on analysis revealed that the down regulation results from a decreased rate of transcription rather than a reduction in the half-life of the RNA. Initiated transcripts in nuclei isolated from RMC2h cells and from RMC2h cells transfected with pWG29 were allowed to continue in the presence of [^{32}P]dUTP under conditions in which no new initiation of RNA synthesis occurs. The amount of labeled RNA synthesized is thus proportional to the rate of transcription. Labeled RNA was used for hybridization to a nitrocellulose filter to which an excess of plasmids containing a 5.2-kb *gag/pol*-specific fragment (Fig. 1), an RSV LTR-specific fragment (Fig. 1), or an actin-specific fragment had been applied (Fig. 4). Densitometric analysis revealed that the amount of RSV LTR-specific RNA is 3-fold reduced and that the amount of MMTV *gag/pol*-specific RNA is 2.2-fold reduced in the RMC2h cells transfected with pWG29 compared with the parental RMC2h cells, whereas the rate of actin transcription remained unchanged. These changes are very similar to the reduction observed in the S1 analysis (2.6-fold reduced) (Table 1), suggesting that a reduced rate of transcriptional initiation is indeed responsible for the observed reduction in steady-state RNA. Changes in the half-lives of MMTV RNAs could also be ruled out by growing RMC2h cells and RMC2hpWG29 cells for increasing lengths of time in the presence of dactinomycin, an inhibitor of RNA initiation. The half-lives of RSV-MMTV transcripts from the two cell lines were essentially identical (data not shown).

These results suggest that pWG29 encodes a factor that acts negatively and in *trans* on transcription from the RSV-MMTV construct. This provides a possible explanation for the relatively low titers of recombinant virus production that we have previously observed, since down regulation of genomic-length mRNA would result in both reduced levels of *gag* structural proteins required for virus production and low levels of packageable RNA.

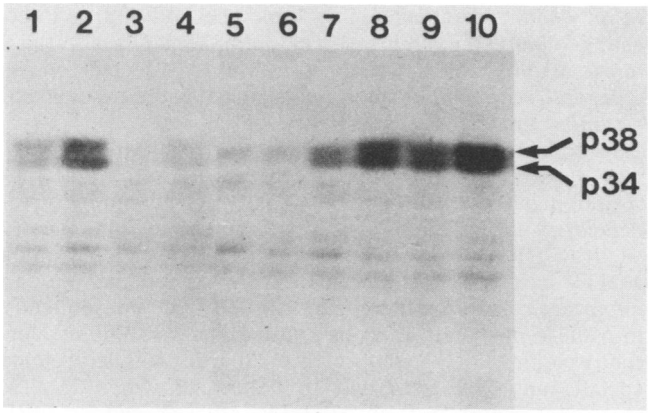

FIG. 2. Western blot analysis of membrane proteins from RMC2h cells transfected with pSV2neo (lanes 1 and 2), pWG29 (lanes 3 and 4), pΔ1 (lanes 5 and 6), pΔ3 (lanes 7 and 8), and pΔ116 (lanes 9 and 10) grown in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 10^{-6} M dexamethasone. An antiserum directed against the mature *gag* protein p28 was used to visualize *gag*-related proteins.

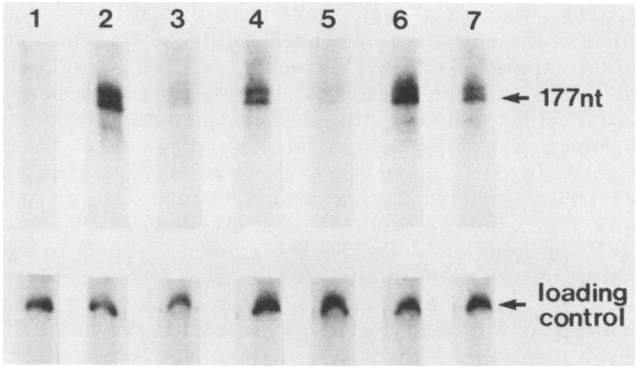

FIG. 3. Total RNAs (40 μg each) isolated from the MMTV producer cell line 2C9 (16) (lane 1), RMC2h cells (lane 2), RMC2h transfected with pWG29 (lane 3), pWG29orf/cla (lane 4), pΔ1 (lane 5), pΔ3 (lane 6), and pΔ116 (lane 7) grown in the presence of 10^{-6} M dexamethasone were hybridized to the 2.0-kb *Pst*I probe shown in Fig. 1 and digested with S1, and the products were separated on a 6% denaturing polyacrylamide sequencing gel. Chimeric RSV-MMTV transcripts protect a 177-nucleotide (nt) fragment.

The putative ORF product has yet to be assigned a function in the viral life cycle. In order to determine whether the ORF product is involved in the observed down regulation of steady-state RNA levels, we introduced a mutation into the ORF of the pWG29 vector construct. A *Cla*I linker was inserted into the *Stu*I restriction site located halfway through the ORF-coding region (Fig. 1; pWG29orf/cla). The insertion of the *Cla*I linker is expected to cause a frameshift, resulting in premature termination of the ORF protein. To verify that frameshifting would occur as expected, the region around the linker insertion site of pWG29orf/cla was sequenced (Fig. 1). The premature termination results in the loss of 10 kDa from the carboxy terminus of the ORF protein(s). After transfection of the pWG29orf/cla plasmid into RMC2h cells, RNA was isolated from a population of stably selected cells and tested by S1 analysis for transcripts initiating at the RSV promoter. In contrast to RMC2hpWG29 cells, the RMC2hpWG29orf/cla cells did not show a down regulation of transcription from the RSV-MMTV construct (Fig. 3, lane 4) (Table 1). This suggests that the ORF product is involved in the in *trans*-negative regulation of mRNA levels in the RMC2h cells.

TABLE 1. Expression of MMTV p34 protein and RSV-MMTV chimeric RNA in transfected RMC2h cells[a]

Transfected plasmid	p34[b]		Dex induction factor[c]	RSV-MMTV mRNA (+Dex)[d]
	−Dex	+Dex		
pSV2neo	100	150	1.5	100
pWG29	23	62	2.7	38
pWG29orf/cla	NS	NS	NS	102
pWG29Δ1	57	57	1.0	27
pWG29Δ3	93	214	2.3	125
pWG29Δ116	179	215	1.2	100

[a] The Western blot in Fig. 2 and the S1 analysis shown in Fig. 3 were densitometrically scanned. NS, Not shown; Dex, dexamethasone.

[b] Expressed as percentages relative to the amount of p34 expressed in RMC2h cells transfected with pSV2neo. −Dex, Without added Dex; +Dex, with added Dex.

[c] Induction factor = p34(+Dex)/p34(−Dex).

[d] Expressed as percentages relative to the amount of RSV-MMTV mRNA detected in RMC2h cells transfected with pSV2neo.

An antiserum, directed against the predicted ORF proteins, has been reported (15). The serum was raised against a hydrophilic, and thus probably antigenic (10), 14-mer peptide. This antiserum did not convincingly reveal *naf* in either Western blotting or immunoprecipitation experiments. However, the MMTV variant used in our experiments carries an amino acid substitution (Thr to Lys) within this 14-mer region. This substitution creates a novel, highly hydrophilic region probably not recognized by the antiserum. We are currently raising antiserum specific for the amino acid sequence of our variant. Alternatively, *naf* may not be expressed at high levels if it is a regulatory protein, making detection difficult.

In an independent experiment designed to adapt the pWG29 vector construct so that it can carry a second heterologous gene of interest, nonessential sequences in the residual *gag/pol* sequences of the pWG29 vector construct were removed. This was achieved by linearization of pWG29 with *Kpn*I followed by controlled digestion with *Bal* 31, treatment with exonuclease VII to create blunt ends, and ligation of *Cla*I linkers. The extent of each deletion was initially determined by restriction mapping, followed by fine mapping using either S1 analysis or direct sequencing (Fig. 1).

While testing whether these constructs retained biological

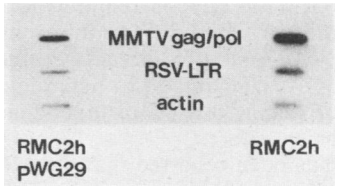

FIG. 4. Transcriptional repression of MMTV mRNA in pWG29-transfected RMC2h cells. Nuclei were prepared from RMC2h cells or pWG29-transfected RMC2h cells, and [^{32}P]UTP-labeled run-on RNA was synthesized. The labeled RNA was extracted and hybridized to 2 μg of a plasmid containing a 5.2-kb *Pst*I restriction fragment from the MMTV *gag/pol* region shown in Fig. 1, a 0.6-kb RSV-LTR fragment, or a 0.6-kb actin-specific fragment immobilized on nitrocellulose filters. The autoradiogram was quantitated by densitometry.

activity, we noticed that, similarly to the ORF mutation, some of these deletions also abolished the down regulation of the expression of MMTV-specific RNA and *gag* proteins in RMC2h cells. Transfection of either pΔ116 (Fig. 3, lane 7) or pΔ3 (Fig. 3, lane 6) into RMC2h cells does not seem to influence transcription from the RSV promoter at all, as assayed by S1 mapping (Table 1). In contrast, transfection with the plasmid that has the smallest amount of *gag* deleted, pΔ1 (Fig. 3, lane 5), results in a fourfold down regulation of RSV transcription levels (Table 1), which is similar to that observed with the wild-type pWG29 (Fig. 3, lane 3) (Table 1). These results implicate the sequences present in pΔ1 but not in pΔ116 or pΔ3 (Fig. 1) as involved in mediating the down regulation of the RSV promoter. These results were confirmed by Western blotting experiments using crude membrane extracts from the same RMC2h cells independently transfected with each of the *gag* deletion plasmids. Again, the level of *gag* protein production from the RSV-MMTV construct in the RMC2h cell lines transfected with pΔ1 (Fig. 2, lanes 5 and 6) is strongly reduced (Table 1) when compared with the pSV2neo-transfected RMC2h cells (Fig. 2, lanes 1 and 2), while RMC2h cells transfected with pΔ3 (Fig. 2, lanes 7 and 8) or pΔ116 (Fig. 2, lanes 9 and 10) show levels of *gag* protein production comparable to that of the pSV2neo-transfected RMC2h cells (Fig. 2, lanes 1 and 2).

Thus, the results obtained using the *gag/pol* deletion mutants of pWG29 further define the *gag* region located between +320 and +646 and/or the *gag/pol* region located between +3626 and +4590 as being involved in the down regulatory effect. These regions may be required for the production of functional ORF, or alternatively they may encode another, as yet unidentified protein. Although the first possibility cannot be ruled out, preliminary experiments have failed to detect the presence of splice acceptor sites within either region. It has been reported that an alternative reading frame in the *gag* gene of RSV encodes a *trans*-acting transcriptional activator (2). However, it seems unlikely that the implicated MMTV *gag* sequences encode a *trans*-acting transcriptional repressor since sequence analysis does not reveal a significant coding potential within the defined *gag* regions in the two alternative reading frames. We are currently cloning ORF cDNAs which may allow us to distinguish between these possibilities.

Although the RSV-MMTV indicator construct itself carries the putative intact ORF-coding region in the 3′ MMTV LTR (Fig. 1A), the RSV-MMTV construct may not direct the synthesis of the ORF product(s) or it may give rise to an inactive form. RMC2h cells express MMTV structural proteins at relatively high levels (18), suggesting that *naf* is not expressed in these cells. This may indicate that, unlike *gag* or *env*, the 5′ MMTV LTR is required for production of ORF-specific mRNA. Support for this hypothesis comes from the observations that constructs designed to express ORF (e.g., MMTV LTR linked to heterologous promoters such as those of simian virus 40 or thymidine kinase) fail to show any biological effect.

Recently, it has been reported that the MMTV ORF is a transactivator of transcription from the MMTV LTR (20) rather than a negatively acting regulator, as reported here. The ORF region has the potential to direct the synthesis of a number of related products (5, 6, 19). We cannot rule out the possibility that two or more products of the ORF region with opposing effects can be synthesized in MMTV-infected cells. In the study mentioned above, the chloramphenicol acetyltransferase gene was placed under the transcriptional control of the MMTV LTR and transfected into noninfected or MMTV-infected rat mammary tumor cells (20). It is interesting to note that in this study chloramphenicol acetyltransferase activity was also reduced in MMTV-infected cells as compared with noninfected cells, suggesting the existence of a *trans*-acting negative regulatory factor.

In conclusion, our results suggest a parallel for MMTV with respect to the complex regulation of retroviral transcription and translation already shown for human immunodeficiency virus (for a review, see reference 14). Clearly such an ORF-mediated regulatory mechanism controlling MMTV expression as described in this paper may play an important role in the life cycle of MMTV and consequently in MMTV-mediated mammary tumorigenesis. We are currently cloning ORF-specific cDNAs in order both to produce specific anti-ORF sera and to determine which MMTV sequences, apart from the 3′ LTR, are required for ORF production.

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