JOURNAL OF VIROLOGY, Sept. 1987, p. 2742–2746
0022-538X/87/092742-05$02.00/0

Vol. 61, No. 9

Two Distinct Sequence Elements Mediate Retroviral Gene Expression in Embryonal Carcinoma Cells

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Received 27 February 1987/Accepted 15 May 1987

Moloney murine leukemia virus (M-MuLV) and M-MuLV-derived retroviral vectors are not expressed in early mouse embryos or in embryonal carcinoma cells. M-MuLV-derived mutants or M-MuLV-related variants which transduce the neomycin phosphotransferase gene can, however, induce drug resistance in embryonal carcinoma cells with high efficiency. In this study we investigated the sequences critical for retroviral gene expression in two different embryonal carcinoma cell lines, F9 and PCC4. We show that two synergistically acting sequence elements mediate expression in embryonal carcinoma cells. One of these is located within the U_3 region of the viral long terminal repeat, and the second one is in the 5′ untranslated region of the retrovirus. The latter element, characterized by a single point mutation, affects the level of stable RNA in infected cells, suggesting a regulatory mechanism similar to that of human immunodeficiency virus in human T cells.

Retroviruses can be used to efficiently introduce foreign genes into the genome of mammalian cells, including germ cells. However, vectors derived from Moloney murine leukemia virus (M-MuLV) are transcriptionally inactive in early embryonic as well as embryonal carcinoma (EC) cell lines, which serve as an in vitro system for the study of gene expression in embryonal stem cells (2, 13, 15, 30, 32). Transcriptional inactivity of retroviruses in stem cells has severely hampered their use for gene transfer into animals (12, 14, 35). It furthermore reveals that embryonic and differentiated cells have different sequence requirements for gene expression and that retroviruses can serve as useful probes for studying the parameters of developmental gene activation on a molecular level.

Virus expression in EC cells appears to be blocked on at least two levels: (i) enhancer sequences located in the long terminal repeat (LTR) of M-MuLV are not functional (11, 16); (ii) a second sequence element 3′ of the LTR, defined by a point mutation in the host range mutant B2, has been shown to be crucial for virus expression in EC cells (4). Another host range variant, myeloproliferative sarcoma virus (MPSV), has been demonstrated to be expressed more efficiently in EC cells than M-MuLV-derived vectors (10, 27). It has been suggested that MPSV expression in EC cells is due to structural differences within the MPSV LTR (10, 27, 31) which have been shown to be responsible for the extended host range for disease induction of MPSV over other murine sarcoma viruses (21, 33, 34). In this study, we have constructed recombinants between M-MuLV, B2, and MPSV to define the sequence elements mediating gene activity in EC cells and investigate the mechanism by which they exert their effect.

MATERIALS AND METHODS

Cell culture. EC cell lines F9 and PCC4 were grown on gelatin-coated plates in Dulbecco modified Eagle medium supplemented with penicillin, streptomycin, and 10% fetal calf serum. NIH 3T3 cells, ψ2 cells (18), and ψ am cells (6) were grown in the same medium except the fetal calf serum was replaced with 10% calf serum. DNA transfections were done by the method of Parker and Stark (22). Infection of cells with virus was done by the method of Cepko et al. (5). G418^r colonies were selected by exposing the cells to the drug beginning 48 h after transfection or infection. The G418 (GIBCO Laboratories) concentration in the culture medium used for selection was 1 mg/ml for 3T3 cells and 0.5 mg/ml for EC cells.

Recombinant DNA methods. All procedures were done essentially as described by Maniatis et al. (17). DNA sequencing was carried out by the method of Sanger et al. (26), using M13 sequencing vectors described by Messing (20). The following plasmids used have been described previously. pSP6*neo* (7) served as template for the in vitro synthesis of an antisense *neo* RNA probe used for the RNA analysis. pXL*neo* and pβ2 124 (7) were radioactively labeled by nick translation (17) and used as DNA hybridization probes for the *neo* gene and the β2 microglobulin gene, respectively.

Quantitative RNA analyses. Total cellular RNA was isolated by the LiCl-urea method (3). An SP6 RNase protection assay (19) was performed with an RNA hybridization probe of 240 bases, which protects the 3′ 168 bases of *neo* RNA (7). The assay was performed as described in reference 7, except that the hybridization was done at 50°C. The protected fragments were separated on 5% polyacrylamide–8 M urea gels.

Recombinant viruses. Recombinant viruses were constructed as follows. A plasmid representing a circularized MP10 provirus containing one LTR (4) was used as a recipient for different restriction fragments from MPSV (33) and B2 (4) proviral DNA. Retrovirus stocks were obtained by transfection of the parental and recombinant plasmids into ψ am cells (6). At 24 h after transfection, supernatants were collected and used to infect ψ2 cells (18). After selection in G418 (1 mg/ml), populations of resistant colonies were obtained and used as producer cells.

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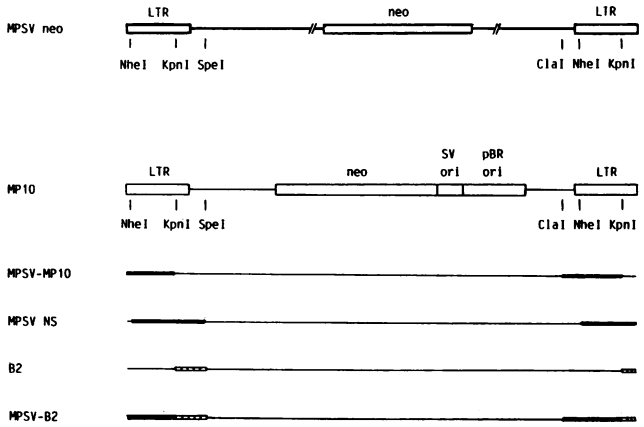

FIG. 1. Restriction maps of MPSV-*neo* (27), MP10 (4), and recombinant derivatives of these proviruses. The position of the *neo* gene (neo) and the origins of replication from simian virus 40 (SV ori) and pBR322 (pBR ori) are indicated. The recombinant viruses are drawn schematically. MPSV sequences are represented by solid bars; B2-derived sequences are indicated as hatched bars.

RESULTS

Construction and characterization of recombinant retroviruses transducing G418 resistance in EC cells. MPSV-*neo* (Fig. 1), an MPSV-derived retroviral recombinant that has a deletion in the *mos* sequences and carries a *neo* gene adjacent to its *gag* sequences, confers G418 resistance to F9 and PCC4 cells with different efficiencies (10, 27) (Table 1). While the absolute titers on a particular cell type vary from one virus stock to the other (due to time of collection, density of producer cells, etc.), the ratio of virus titers on F9 or PCC4 cells relative to 3T3 cells was constant between different experiments. Thus, MPSV-*neo* titers on F9 and PCC4 cells were about 10- and 1,000-fold lower, respectively, than on 3T3 cells (Table 1). Because F9 and PCC4 cells were infected at equal efficiencies, the viral genome is likely to be more efficiently expressed in F9 than in PCC4 cells (10, 27). The B2 mutant virus (4) was obtained after proviral DNA rescue from a rare G418-resistant F9 colony which was selected after infection with the M-MuLV-derived *neo*-transducing virus MP10 (Fig. 1) (4). The *neo* gene in MP10 is under the control of the M-MuLV LTR and is not expressed in EC cells (Table 1). The phenotypic difference between the B2 and MP10 viruses is due to a single base change at position 607 (4) (Fig. 2), which is outside the LTR in the 5′ untranslated region of the virus. The B2 mutant induces G418-resistant colonies on both F9 and PCC4 cells equally well and at least 100 times more efficiently than the parental MP10 virus. Compared with MPSV-*neo*, the B2 titers are 10 times lower on F9 cells but 10 times higher on PCC4 cells. These data suggested to us that different sequence elements might be responsible for the different expression of MPSV-*neo* and the B2 virus in EC cells.

To map the sequences within the MPSV genome critical for expression in EC cells, we derived recombinants between MPSV and MP10. In the first recombinant, MPSV-MP10 (Fig. 1), we replaced the U_3 region from MP10 with the corresponding sequences from MPSV. A second recombinant, MPSV-NS, contains a 695-base pair *Nhe*I-*Spe*I fragment from MPSV in place of the respective fragment from MP10. This fragment contains most of U_3, all of R and U_5, and an additional 137 base pairs of 5′ untranslated leader sequences outside of the LTR; it was chosen because it spans the region essential for expression of the B2 virus in EC cells. When the MPSV-MP10 recombinant virus was tested for its ability to confer G418 resistance to EC cells, the U_3 region from MPSV was found to be insufficient to allow for *neo* expression (MPSV-MP10; Table 1). In contrast, when the 5′ untranslated region from MPSV was included, G418-resistant colonies appeared at efficiencies similar to those with the original MPSV-*neo* virus (MPSV-NS; Table 1). This indicated that, as with the B2 virus, MPSV contains a sequence element essential for expression in EC cells in the 5′ untranslated region of the virus.

To investigate a possible enhancing function of the MPSV U_3 region on gene expression in EC cells, we constructed a third recombinant virus, MPSV-B2 (Fig. 1). This virus contains the MPSV U_3 region (*Nhe*I-*Kpn*I) and the B2

TABLE 1. G418^r titers on EC cells and fibroblasts[a]

Virus	G418^r colonies per ml of virus			Titer (EC/3T3)	
	3T3	F9	PCC4	F9/3T3	PCC4/3T3
MPSV-*neo*	1.2×10^6	5.0×10^4	6.3×10^2	0.042	0.0005
	7.5×10^5	5.0×10^4	8.0×10^2	0.066	0.001
MP10	3.5×10^5	0	0	0	0
	6.0×10^4	0	0	0	0
MPSV-MP10	3.6×10^4	0	0	0	0
	2.2×10^4	0	0	0	0
MPSV-NS	7.5×10^4	3.5×10^3	1.1×10^2	0.046	0.0015
	1.5×10^5	1.7×10^3	8.5×10^2	0.011	0.005
B2	2.0×10^6	5.0×10^3	6.3×10^3	0.0025	0.0031
	1.5×10^5	4.3×10^2	4.0×10^2	0.0036	0.0033
MPSV-B2	3.8×10^4	4.3×10^3	3.5×10^3	0.11	0.092
	2.0×10^4	1.7×10^3	1.2×10^3	0.085	0.065

[a] Supernatants from virus-producing cells were used to infect 3T3, F9, and PCC4 cells. The selection conditions applied were 1 mg of G418 per ml in Dulbecco modified Eagle medium supplemented with 10% calf serum for 3T3 cells and 0.5 mg of G418 per ml in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum for F9 and PCC4 cells. Numbers given represent results from individual experiments. For each virus, titers were determined in at least two independent experiments.

mutation (*Kpn*I-*Spe*I) in an MP10 backbone. MPSV-B2 induces high titers of G418-resistant colonies (only 10-fold lower than on 3T3 cells) on both F9 and PCC4 cells (Table 1). Relative to the titers on 3T3 cells, these titers are about 10 times higher than those of the B2 virus on both EC cell types. Therefore, our results permit the following conclusions. (i) The MPSV U_3 region, although not sufficient to mediate G418 resistance in EC cells, enhances viral gene expression by one order of magnitude as compared with the M-MuLV U_3 region. (ii) The MPSV LTR functions equally well in F9 and PCC4 cells when combined with the B2 mutation. Thus, the more efficient expression of MPSV-*neo* in F9 than in PCC4 cells (Table 1) cannot be due to sequences within the U_3 region of the LTR. (iii) Our results indicate that at least two distinct sequence elements, which act synergistically, are important for retroviral gene expression in EC cells.

Nucleotide sequence of the sequence element essential for expression in EC cells. To investigate the structure of the sequence element located in the 5′ untranslated region of MPSV, we determined the nucleotide sequence of the *Kpn*I-*Spe*I fragment from this virus and compared it to the sequence of the corresponding region in MP10 and B2 (Fig. 2). The comparison revealed six sequence differences between MPSV and MP10 in this region: four point mutations, one single-base-pair deletion, and one 2-base-pair substitution. Three changes are located within the R-U_5 region of the LTR and two changes are located 3′ of the splice donor site. One sequence alteration was found at position 611, where a C residue in MP10 is replaced by two T residues in MPSV. This change maps close to the single point mutation that distinguishes B2 from MP10 at position 607. However, none of the sequence differences between MPSV and MP10 overlaps the B2 mutation.

The B2 mutation affects the level of stable viral RNA in EC cells. To study the mechanisms by which the B2 mutation affects virus expression, we measured the levels of stable RNA produced by different integrated proviruses in different cell types. 3T3 and F9 cells were infected with MPSV-B2 and MPSV-MP10, respectively, and passaged twice in nonselective media before total cellular RNA and DNA were isolated. The RNA was analyzed for the presence of *neo*-specific sequences, using a quantitative SP6 RNase protection assay (19). The efficiency of infection was monitored by Southern blot analyses of DNA isolated from the infected cells. This allowed us to normalize the RNA concentration to the copy number of stably integrated proviruses. Figure 3 shows the result of this experiment. The RNA was analyzed with an RNA probe which, when hybridized to viral RNA, protects the 3′-terminal 168 nucleotides of the *neo* gene from RNase digestion (7). This fragment can be readily detected in 3T3 cells infected with either virus and in F9 cells infected with MPSV-B2 (Fig. 3a). Densitometric analyses, normalized to provirus copy number (see below), revealed that F9 cells infected with MPSV-B2 contain about 10 times more viral RNA than F9 cells infected with MPSV-MP10, while the amounts of viral RNA found in 3T3 cells infected with either virus were equal. Thus, the B2 mutation affects the level of stable RNA in infected F9 cells but not in 3T3 cells. Furthermore, the amount of virus-specific RNA found in MPSV-B2-infected F9 cells is, per template, about 50 times lower than in 3T3 cells infected with the same virus, consistent with the hypothesis that the promoter or enhancer or both in the retroviral U_3 region functions much better in the 3T3 cells. Figure 3b shows the analysis of the proviral copy number in the different infected cell populations. The signal

```
                                                  R◄┬►U5
480  GGTACCCGTG TATCCAATAA ACCCTCTTGC AGTTGCATCC GACTTGTGGT
                  T                                  C

530  CTCGCTGTTC CTTGGGAGGG TCTCCTCTGA GTGATTGACT ACCCGTCAGC

               U5◄┐                 A
580  GGGGTCTTTC ATTTGGGGGC TCGTCCGGGA TCGGAGACCC CTGCCCAGGG
                                     TT
                              SD
630  ACCACCGACC CACCACCGGG AGGTAAGCTG GCCAGCAACT TATCTGTGTC

680  TGTCCGATTG TCTAGTGTCT ATGACTGATT TTATGCGCCT GCGTCGGTAC TAGT
                  G              T
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FIG. 2. Nucleotide sequence of the *Kpn*I-*Spe*I fragment from the MP10 provirus compared with MPSV and B2. The differences in MPSV are given below the MP10 sequence. Four single point mutations and a single-base-pair deletion (–) are indicated. At position 611 a C residue in MP10 is replaced by two T residues. The single point mutation distinguishing the B2 virus from MP10 at position 607 is boxed in above the MP10 sequence. The limits of the R region and the U_5 region are shown above the sequence, as well as the position of the splice donor site (SD). The $tRNA^{Pro}$ primer binding site is emphasized by underlining. The nucleotide numbering refers to the 5′ end of the LTR as position 1.

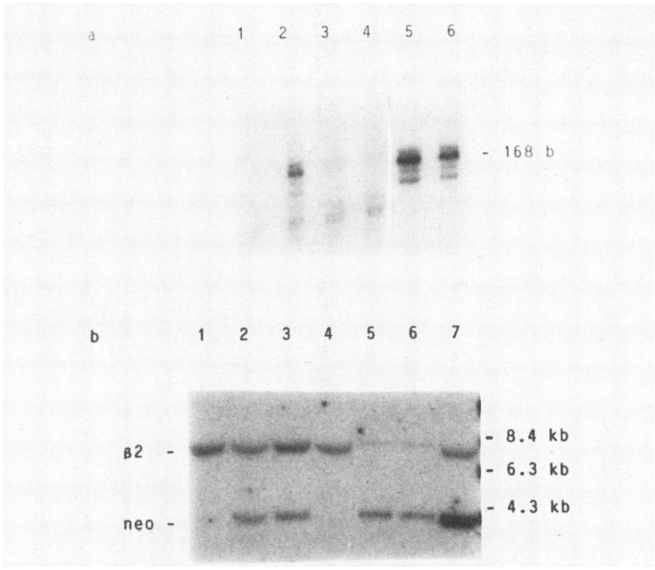

FIG. 3. Quantitative analysis of viral RNA in infected F9 and 3T3 cells. A total of 10^5 cells of each cell type were plated and infected with MPSV-B2 and MPSV-MP10 by repeated exposure to fresh virus-containing media for several days. After infection the cells were grown for two passages in nonselective media before total cellular RNA and DNA were prepared. The amount of viral RNA was determined by using quantitative SP6 analysis (19) (a), and the efficiency of infection was monitored by Southern blot hybridization (b). Lanes 1 to 3, F9 cells; lanes 4 to 6, 3T3 cells; lanes 1 and 4, uninfected; lanes 2 and 5, MPSV-B2-infected cells; lanes 3 and 6, MPSV-MP10-infected cells. (a) RNA analysis. The probe used protects the 3′ 168 bases of *neo* RNA (7). The amount of total cellular RNA used was 10 (lanes 1 to 4) and 1 (lanes 5 and 6) μg. Lanes 1 to 4 are taken from a 15-h exposure, and lanes 5 and 6 are from a 1.5-h exposure of the same gel. (b) DNA analysis. A 10-μg (lanes 1 to 4, 7) or 2-μg (lanes 5 and 6) amount of cellular DNA was digested with *Sac*I and separated on a 0.8% agarose gel. After transfer to a nylon membrane, the DNA was hybridized to a mixture of radioactively labeled β2 microglobulin and *neo*-containing plasmids. The *neo* probe detects a unit-length proviral fragment (neo), while the β2 microglobulin probe detects the endogenous gene (β2), serving as an internal standard. Lane 7 represents a single-copy control for the MP10 provirus in F9 cells. b, Bases; kb, kilobases.

obtained with a 4.0-kilobase virus band detected by a *neo* probe was compared with the intensity of an 8.0-kilobase band detected by a β2 microglobulin probe, which served as an internal standard. This analysis revealed that, although both cell types had been infected with the same virus stock and using the same infection protocol, the 3T3 cells were infected about five times more efficiently than F9 cells. When corrected for infection efficiency, our results suggest that the MPSV-B2 provirus induces drug resistance in EC cells nearly as efficiently as in 3T3 cells.

DISCUSSION

In this study we have used host range variants to study the control of retrovirus expression in EC cells. While M-MuLV-derived vectors are poorly expressed in EC cells, the B2 point mutation (4) induced drug resistance equally in the two EC cell lines, F9 and PCC4. MPSV-derived vectors differ from the B2 mutation as they induce drug resistance efficiently in F9 cells but only with a low frequency in PCC4 cells (10, 27). We therefore constructed recombinants between the three host range variants in an effort to define sequence elements important for retrovirus expression in EC cells.

This study identified two sequence elements important for retrovirus expression in EC cells. One element is located in the U_3 region of the LTR and enhances gene expression at least 10-fold in F9 and PCC4 cells relative to 3T3 cells. The absolute titers of stocks of MPSV recombinants showed relatively low titers on 3T3 cells. We attribute this to normal variations in collection of virus stocks rather than to a decreased efficiency of the MPSV LTR in 3T3 cells. This is supported by the observation that the MPSV LTR functions two- to fourfold better than the M-MuLV LTR in F9 cells but comparably in 3T3 cells, as determined by chloramphenicol acetyltransferase assay (E. Barklis and R. Jaenisch, unpublished results; N. Speck, personal communication). Also, using different virus stocks, we have observed that 3T3 cells are infected with approximately equal efficiency by B2 and MPSV-B2 viruses, as determined by quantitation of proviral DNA in unselected cells (data not shown). In any case, the MPSV U_3 region is not sufficient to permit *neo* expression, as measured by the formation of G418-resistant colonies in EC cells. A second sequence element located in the 5′ untranslated region of the virus is essential for this function. Transfer of the MPSV U_3 region to the MP10 construct (MPSV-MP10) is not sufficient to permit *neo* expression in EC cells, but addition of the MPSV 5′ untranslated region permits *neo* expression in MPSV-NS-infected EC cells (Table 1). It may be argued that the 167-base pair MPSV *Cla*I-*Nhe*I DNA fragment which is absent in MPSV-NS but present in MPSV-MP10 exerts an inhibitory influence on MPSV-MP10 expression in EC cells. We feel that this is unlikely, since MPSV-*neo* contains this MPSV segment and is expressed in EC cells in a fashion similar to MPSV-NS (Table 1). Thus, a sequence element defined by a single point mutation in the B2 virus and multiple base changes in the MPSV element determines retrovirus expression in EC cells. Also, this element is responsible for the apparent cell type specificity of MPSV-*neo* that is permitting the establishment of G418 resistance in F9 but not PCC4 cells. Although we do not know whether the mutations in B2 and MPSV affect expression in EC cells by the same mechanism, we have shown that the B2 mutation acts synergistically with the enhancer element in the U_3 region of the LTR. Furthermore, we have established that the B2 mutation enhances the level of stable RNA present in F9 cells by about 10-fold, while it has no enhancing effect in 3T3 cells. The 10-fold enhancement of stable RNA levels contrasts with an approximately 1,000-fold stimulation in the formation of G418-resistant colonies in F9 cells. One possible explanation is that the level of expression in F9 cells might be close to a threshold level required to render F9 cells resistant to G418. Alternatively, the point mutation in the B2 virus could affect the efficiency of translation as well. In this case it could be significant that the B2 mutation is located in the $tRNA^{Pro}$ primer binding site (Fig. 2) of the viral RNA, potentially destabilizing a hybrid between viral RNA and $tRNA^{Pro}$ which is formed in the normal retrovirus life cycle. However, because in MPSV the $tRNA^{Pro}$ primer binding site is not mutated, mechanisms other than tRNA binding would have to be involved in translational regulation as well.

There are precedents for the involvement of 5′ untranslated sequences in the regulation of retroviral gene expression. The regulation of expression of human immunodeficiency virus, the virus causing acquired immune deficiency syndrome (AIDS), involves a *cis*-acting region close to the 5′ end of the RNA and a *trans*-acting function encoded by the viral genome (1, 23, 24, 28). Recently, it has been shown that this regulation affects the level of stable RNA as well as the efficiency of translation (8). Similarly, bovine leukemia virus contains a control element for gene expression within the 5′ untranslated region (9). The B2 virus represents the first example of a mutation in this novel type of regulatory element.

The nonexpression of M-MuLV in early embryonic cells has hampered the use of retroviral vectors as expression vectors in gene transfer experiments into transgenic mice (12, 14, 35). The generation of viral mutants, as described here, or the use of internal promoters (25, 29, 36) may be two alternative strategies to overcome this restriction. Because EC cells can be grown as homogeneous cell populations, viral mutants can easily be selected. It is possible that cellular mechanisms permitting virus expression in EC cells are of general significance and are similar to those operating in stem cells of other lineages such as the hemopoietic system.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants HD-19105 from the National Institutes of Health and P01-CA 38497 from the National Cancer Institute. H.W. and W.O. were supported by grants from the Deutsche Forschungsgemeinschaft.

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