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Different Activities of Viral Enhancer Elements before and after Stable Integration of Transfected DNAs

FAYTH K. YOSHIMURA[1]* AND KAREN CHAFFIN[2]

Departments of Biological Structure[1] and Biochemistry,[2] University of Washington, Seattle, Washington 98195

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Analysis of the RNA and DNA levels of a selectable gene linked to a murine retroviral enhancer demonstrated a correlation between RNA levels and tissue-specific enhancer activity during transient expression in T cells but not in stably transformed cell lines.

Enhancer elements, which are important in the transcriptional regulation of many viral and cellular genes, can function in a tissue-specific manner (1, 2, 5, 11, 18). Enhancer activity in various tissues has been observed for sequences which reside in the long terminal repeats (LTRs) of a number of different murine leukemia viruses (MLVs). It is thought that the tissue-specific activities of MLV enhancer elements contribute to the specific tropism and transforming properties of these viruses (3, 16, 19).

A class of recombinant MLVs referred to as mink cell focus-forming (MCF) viruses is involved in the generation of thymic lymphomas in certain mouse strains (4, 15). A parental virus of the MCF MLVs, known as Akv MLV, does not possess this neoplastic property. By using a stable transformation assay dependent on the expression of a gene conferring neomycin resistance (Neo^r), we previously demonstrated that the MCF-13 enhancer has a significantly greater activity than the Akv enhancer in murine T lymphoid cells (19). Although other *cis*-acting signals within the LTR may contribute to this activity, we have attributed this effect to enhancerlike sequences because we obtained similar results with the LTR in either orientation. Enhancers have the ability to function in an orientation-independent manner. In contrast, the activities of these two retroviral enhancers, as well as the simian virus 40 (SV40) enhancer, were comparable in murine fibroblasts. To understand the mechanism by which the MCF-13 MLV enhancer element is able to function more efficiently in T lymphoid cells than in fibroblasts, we have analyzed the expression of the Neo^r gene both transiently and in stable lines of both T lymphoid and fibroblastic cells.

In our previous study we demonstrated the cell-type-specific activity of the MCF-13 enhancer by determining the transfection efficiencies of plasmid clones containing the selectable Neo^r gene and various viral enhancers. In this study we wished to more rigorously examine whether the MCF-13 enhancer was functioning by an increase in transcription, since other known enhancers have been shown to work by this mechanism.

To do this we analyzed the levels of Neo^r transcripts in SL-3 cells in a transient assay with the different plasmid clones depicted in Fig. 1. Two-thirds of the transfected cells were used to isolate total RNA, which was analyzed by dot blot hybridization as previously described by Kafatos et al. (10). A ^{32}P-labeled DNA fragment containing only Neo^r sequences was used as a hybridization probe. To correct for the variation in uptake of DNAs by cells, we determined the

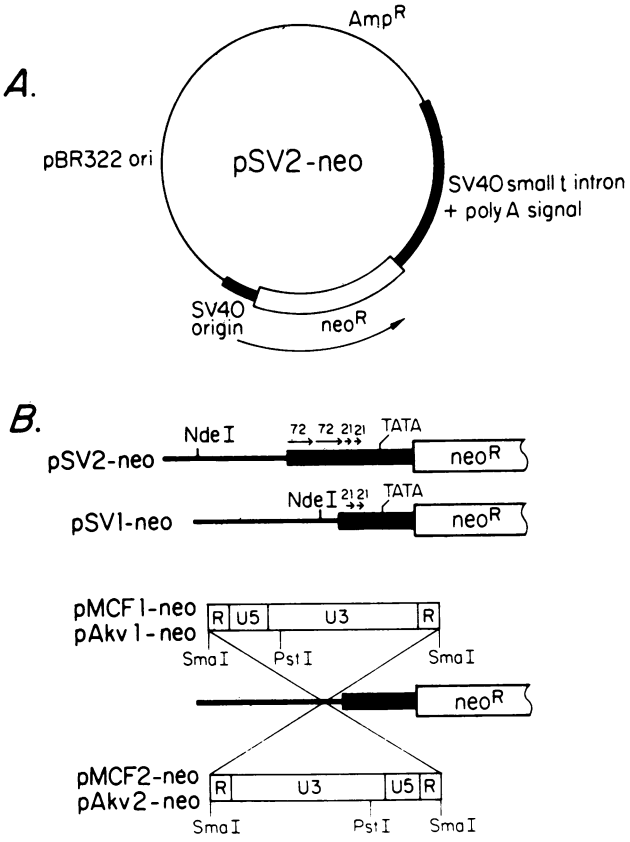

FIG. 1. Plasmid constructs used for transfection experiments (taken from reference 19). (A) pSV2-neo (17) is a pBR322 plasmid that contains the SV40 origin of replication, which includes the repeated 72-base-pair enhancer sequence, the 21-base-pair repeat, and the promoter (TATA) for early viral genes, upstream of the Tn*5* phosphotransferase gene (Neo^r) encoding neomycin resistance (also shown in panel B). The SV40 small-t intron and the poly(A) addition signal have been placed 3′ to this gene. This plasmid also contains a bacterial origin of replication and a gene encoding ampicillin resistance (Amp^r). (B) pSV1-neo (6) is a construct, derived from pSV2-neo, which is missing all but 22 base pairs of the 72-base-pair repeated sequences. pMCF1 and pAkv1-neo have the LTR fragment from the MCF-13 or Akv MLV, respectively, inserted into the *Nde*I site in pSV1-neo in the same orientation as transcription of the Neo^r gene. Constructs with the same LTR fragments inserted in the opposite orientation are pMCF2 and pAkv1-neo. Restriction endonuclease sites (*Pst*I and *Sma*I) which were used in the generation of the LTR fragments are shown.

* Corresponding author.

TABLE 1. Neo^r RNA and DNA in SL-3 cell lines[a]

Cell line	RNA copy no./cell	DNA copy no./cell	RNA/DNA copy no. ratio
pSV1neo-1	6	5	1
pSV1neo-2	5	11	0.4
pSV1neo-5	16	7	2
pSV1neo-6	ND[b]	34	ND
pSV2neo-1	7	6	1
pSV2neo-2	12	7	2
pSV2neo-3	10	9	1
pSV2neo-7	ND	6	ND
pMCF1neo-1	85	15	6
pMCF1neo-2	162	14	12
pMCF1neo-3	165	25	7
pMCF1neo-8	ND	67	ND
pMCF2neo-1	370	400	1
pMCF2neo-2	119	36	3
pMCF2neo-3	89	30	3
pMCF2neo-9	ND	110	ND
pAkv1neo-2	48	7	7
pAkv1neo-4	13	6	2
pAkv1neo-5	12	6	2
pAkv1neo-6	ND	63	ND
pAkv2neo-1	163	11	15
pAkv2neo-2	39	17	2
pAkv2neo-3	56	183	0.3
pAkv2neo-4	ND	58	ND

[a] Densitometric tracings were made of DNA and RNA dot blots with an LKB model 2202 laser densitometer and recording integrator. Known amounts of control DNA (2.5 to 20 μg of pSV1neo plasmid DNA) applied to each dot blot filter were used to calculate the amounts of Neo^r DNA and RNA per cell for each cell line. Cell lines had been transfected with the indicated plasmids and grown in media containing G418. Copy numbers were calculated by assuming that there were 12 pg of cellular DNA and 7 pg of total cellular RNA per mouse T lymphocyte, based on our recoveries.

[b] ND, Not determined.

amount of extrachromosomal DNA for each plasmid by using a modified Hirt protocol (8) involving both dot blot and Southern blot analyses (data not shown).

We observed that the transient expression of plasmids containing the MCF-13 LTR in either orientation (pMCF1neo and pMCF2neo; Fig. 1) resulted in an RNA-to-DNA ratio which was 6- to 13-fold greater than the ratios of the other enhancer-containing plasmids. These results corroborated our previous observations with the same clones for which we used a stable transformation assay instead (19). Transient assays of RNA levels have also been successfully used by other laboratories to demonstrate the cell-type-specific activity of other enhancer elements (1–3, 5).

To determine the effect of the MCF-13 enhancer on transcription in stably transformed lines of T lymphoid cells, we calculated the copy number of total Neo^r RNAs in cloned cell lines transfected with the same plasmids used in the transient assay experiments. RNA copy numbers were again determined by dot blot analysis. The copy numbers of Neo^r transcripts per cell for a number of randomly chosen SL-3 cell lines independently transfected with each plasmid are summarized in Table 1. Copy numbers were calculated from densitometric tracings of autoradiographs (data not shown). The data in Table 1 demonstrate that we were not able to detect a correlation between RNA copy number and enhancer activity in SL-3 cells. For example, line pAkv2neo-1 had more Neo^r RNA per cell than did most of the pMCFneo cell lines. Other lines, such as pAkv1neo-2 and pAkv2neo-3, also had levels of RNAs which did not differ from most of the pMCFneo lines by more than 1.5- to 3.3-fold. These differences could not account for the greater MCF-13 enhancer activity we observed from the transient assays or the stable transformation experiments. This was surprising because of the results from the transient assay which demonstrated a correlation between transcript levels and enhancer activity and because of observations by others showing that enhancers function by increasing transcription (11). Moreover, this observation did not appear to be unique for T lymphoid cells, since we also detected this lack of correlation for cloned fibroblast cell lines that were similarly generated and analyzed (Table 2).

We attempted to account for the lack of transcriptional activation by the MCF-13 enhancer in T cells by examining the copy number per cell of transfected DNA for each of the same cell lines (Tables 1 and 2). Although previous studies have shown that there is usually a lack of correlation between number of integrants and enhancer or gene activity (7, 13), this possibility could not be a priori discounted for our system, which had not been previously examined. The calculations of DNA copy numbers by dot blot analyses, however, still could not account for the different levels of Neo^r RNAs observed in these cells. The data are also presented in Tables 1 and 2 as the ratios of RNA-to-DNA copy numbers.

The presence of aberrantly sized Neo^r transcripts, which

TABLE 2. Neo^r RNA and DNA in NIH 3T3 cell lines[a]

Cell line	RNA copy no./cell	DNA copy no./cell	RNA/DNA copy no. ratio
pSV1neo-1	2,800	397	7
pSV1neo-2	462	243	2
pSV1neo-3	412	99	4
pSV1neo-4	ND[b]	19	ND
pSV2neo-1	260	6	43
pSV2neo-2	84	6	14
pSV2neo-4	196	6	33
pSV2neo-5	ND	2	ND
pMCF1neo-1	250	37	7
pMCF1neo-3	382	26	15
pMCF1neo-6	92	13	7
pMCF1neo-7	ND	11	ND
pMCF2neo-3	150	11	14
pMCF2neo-4	670	100	7
pMCF2neo-5	454	45	10
pMCF2neo-6	ND	15	ND
pAkv1neo-1	1,342	56	24
pAkv1neo-2	662	75	9
pAkv1neo-4	1,218	75	16
pAkv1neo-8	ND	16	ND
pAkv2neo-1	644	10	64
pAkv2neo-2	934	41	23
pAkv2neo-5	88	1	88
pAkv2neo-9	ND	23	ND

[a] Densitometric tracings and calculations were performed for cell lines as described in footnote *a* to Table 1. A 14-pg amount of total cellular RNA per mouse fibroblast based on our recoveries was used to calculate RNA copy numbers per NIH 3T3 cell.

[b] ND, Not determined.

TABLE 3. Copy numbers of Neo^r 2.35-kilobase RNA in SL-3 and NIH 3T3 cell lines[a]

Cell line	RNA copy no./ cell
SL-3	
pMCF1neo-1	70
pMCF1neo-3	80
pMCF2neo-1	300
pMCF2neo-3	30
pAkv1neo-2	44
pAkv2neo-3	50
NIH 3T3	
pSV1neo-1	166
pSV1neo-3	101
pSV2neo-1	14
pSV2neo-2	80
pMCF1neo-1	69
pMCF2neo-3	19
pMCF2neo-4	72
pAkv1neo-1	98
pAkv1neo-2	89
pAkv2neo-2	152
pAkv2neo-5	1

[a] Calculated from densitometric tracings of autoradiographs. Copy numbers were determined from Northern blots of total cellular RNA hybridized with a Neo^r-specific probe.

are not detectable by dot blot analysis, could help to explain our inability to observe an increase in transcription dependent on enhancer activity. We, therefore, determined the copy number of Neo^r transcripts of the correct size (2.35 kilobases) in each of the cell lines by Northern blots. We analyzed total cellular RNAs by electrophoresis through denaturing agarose gels cast in 2.2 M formaldehyde by the precedure of Maniatis et al. (14). Copy numbers of the normal Neo^r transcript per cell were calculated from densitometric tracings of autoradiographs of the Northern blots (Table 3). These values again showed a lack of correlation between enhancer activity and transcriptional potentiation.

One interpretation of our results is that the chromosomal site into which an enhancer integrates can generally exert a greater influence on the transcriptional activation of a gene than an adjacent enhancer element can. Similar types of chromosomal position effects have been previously observed for retroviral integration into mouse embryos by Jaenisch and co-workers (9), who have shown that the chromosomal site of integration was often the determining factor for proviral expression. Other examples include studies of cells transformed by the SV40 early region, where despite increases in transformation frequencies in the presence of a murine sarcoma virus LTR included with the SV40 sequences, there was no concomitant increase in T antigen levels in cells (12).

We have speculated about a mechanism which could account for the difference in the transcriptional activities we have observed before and after stable integration of the enhancer. This mechanism includes as a first step the formation of a primary stable transcriptional complex which is enhancer dependent and which occurs before chromosomal integration of transfected DNA. The role of the enhancer in the formation of this complex would be to increase the rate or probability (or both) of its occurrence. Once integration of this activated complex has taken place, however, the chromosomal site of integration would then exert a stronger influence on the rate of transcription of the gene. One possible effect of the chromosomal site which can be envisioned is to influence the rate of loading of RNA polymerase onto the gene, which in turn would affect the rate of transcription. In this model the enhancer affects the rate of formation of stable transcriptional complexes, but the chromosomal site determines the degree of RNA polymerase density along the gene. Both would be important in determining rates of transcription.

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