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A correlation between dexamethasone inducibility and basal expression levels of retroviral vector proviruses

Mogens Duch, Kirsten Paludan, Jette Lovmand, Lene Pedersen, Poul Jørgensen and Finn Skou Pedersen*

Department of Molecular Biology, University of Aarhus, C.F.Møllers Allé, Bldg. 130, DK-8000 Århus C, Denmark

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ABSTRACT

Identical transcription units inserted at different positions of mammalian chromosomes may vary widely in transcriptional activity. We have used a set of ten cell clones with random unselected single integrations of retroviral vectors to study such position effects. The vector used carries a *neo* gene driven by the Akv murine leukemia virus long terminal repeat that has only a weak promoter – enhancer activity in the target cell, the lymphoid cell line L691. Under transient expression conditions, the strength of the Akv promoter – enhancer in the L691 cells is increased by dexamethasone. In cell clones with single vector integrations, a correlation is observed between the non-induced expression levels and the degree of dexamethasone induction. The strongest relative induction is found for the integrated vectors with the lowest non-induced expression levels and approaches the inducibility under transient expression. These results indicate that expression levels are composed of distinct contributions from the integrated vector and from the site of integration and are best explained in terms of a model in which the sites of chromosomal integration exert variable positive enhancer effects upon vector transcription.

INTRODUCTION

Studies of transient expression after gene transfer into mammalian cells have provided detailed insight into promoter–enhancer functions. Additional levels of control operate on genes stably inserted into a chromosome. In most cases the magnitude of these higher order regulatory effects vary according to the actual positions of the gene, and stable insertions of a transferred gene result in clones with a wide distribution of expression levels.

Although details of the mechanisms behind the effects of chromosomal positions on gene expression in mammals are largely unknown (1) it appears that several types of regulatory interactions may be involved. Enhancer or promoter elements at a chromosomal site may affect expression of an inserted gene (2, 3, 4, 5, 6). By a distinct mechanism, some chromosomal positions may result in variable frequencies of shut-down of expression of the inserted gene in a process that may be associated with DNA-methylation (7, 8). Regulatory sequences that may contribute to position independent expression of a transferred transcriptional unit have been identified by biochemical or genetic means in mice (9) or chicken (10).

Retroviruses and retroviral expression vectors integrate as single well-defined transcription units at a very large number of sites in the genome of the host cell (11, 12, 13). The use of retroviral vectors and retroviral packaging lines permits single-cycle transductions at high titers of vector particles (14) and therefore identification of transduced cell clones by screening for the presence of vector sequences, thereby avoiding the bias introduced by selecting for vector expression (15). We previously observed a wide distribution of proviral expression levels among such non-selected cell clones with single copies of identical murine leukemia virus-derived expression vectors. Using either a strong (SL3-3) or a weak (Akv) transcriptional enhancer in the lymphoid L691 cell line, the widest span of expression levels was observed in case of vectors with the weaker Akv enhancer (16).

This material therefore allows systematic studies of the effect of chromosomal DNA environment upon the activity of a well-defined transcription unit. The specific role of the flanking DNA in affecting the expression level of these integrated vectors might be further studied by gene transfer studies after molecular cloning. Since, however, not all aspects of higher order gene regulation might be revealed by gene transfer assays for regulatory functions of DNA, we decided to assess the contribution from the sites of integration by altering the enhancer strength of the inserted vectors, located in their original chromosomal positions. The steroid hormone dexamethasone (Dex) acts in complex with the glucocorticoid receptor as a *trans*-regulator of enhancer strength through binding to glucocorticoid responsive elements (17). Such sequence elements are present in the enhancer region (U3) of the Akv murine leukemia virus (18) and Dex has been found to increase expression driven by the Akv enhancer–promoter in some lymphoid cell lines (19).

We here report that Dex stimulates expression mediated by the Akv promotor enhancer in L691 cells and that the relative

* To whom correspondence should be addressed

inducibility of the integrated vectors correlates inversely with the basal expression levels. For the integrated vectors with lower expression levels the response resembles that observed under transient expression conditions, whereas the high expressors show a smaller relative response. We discuss our results in terms of a model for variable stimulatory effects of the chromosomal integration position upon a retrovirally transduced transcription unit.

MATERIALS AND METHODS

Vectors

The retroviral transmission vectors Akv-neo (Fig. 1.) and SL3-3-neo and the vectors pAkv6-cat, and pL6-cat used for transfection and transient expression studies have been described previously (20, 21, 16).

Cell lines

The L691 cell line (22) is a non retrovirus-producing radiation-thymoma cell line of a C57L mouse. Cell clones of L691 containing single integrations of Akv-neo vector proviruses (clones A6, A36, A9, A21, A29, A15, A19, A10, A11 and A30,) are from the sets of cell clones described previously (16). Cultures were free of replication competent viruses as no helper virus could be detected in the infected populations by co-cultivation with NIH 3T3 cells (16).

Cell culture

Cells were grown in suspension in RPMI 1640 medium-10% newborn calf serum. To elimimate non-expressors that might have arisen during subcloning due to inactivation processes, the cell clones were grown in medium with 0.4 mg G418 per ml for one week before Dex induction. This weak selection will not eliminate low expressors nor select for strong expressors and therefore does not introduce a bias in the populations (15, 23). G418 was obtained from Sigma Chemical Co,. St Louis, MO.

Electroporation

DNA was transferred into L691 cells by electrotransfection using a gene pulser apparatus with a capacitance extender (Bio-Rad, Richmond, CA). Electroporation was done in 0.4 cm cuvettes and 0.8 ml Dulbecco's Modified Eagle's Medium (Biochrom, Berlin) without serum, voltage was set at 450 V and capacitance at 125 μF. For measurement of transient expression 30 μg plasmid DNA of pAkv6-cat or pL6-cat were electroporated into 2×10^7 L691 cells. After 44 h, the cells were divided into two subcultures. Dexamethasone (Sigma, St Louis, MO.) was added to one of the subcultures at a concentration of 1 μM. Cells were harvested 4 h later and RNA extracted as described below.

RNA purification

For RNA dot-blot measurement 1.5 ml cultures (1×10^6 cells/ml) were collected by centrifugation and total RNA extracted from the pellets by the method of Chomczynski and Sacchi (24) using 0.5 ml of acid guanidinium thiocyanate buffer. For Northern blots 40 ml of electroporated L691 cells were collected in 50 ml tubes (NUNC, Roskilde, DK) and total RNA extracted using 5.0 ml of acid guanidinium thiocyanate.

Polymerase chain reaction and DNA-sequence analysis

The nucleotide sequences of the direct repeat containing enhancer region in U3 of the integrated retroviral vector proviruses were determined after amplification by polymerase chain reaction (25, 26). Oligonucleotides, their localization (Fig. 1.) and the conditions used were as previously described (16) except that extension times were changed to 3 min and the Taq polymerase used was from Stratagene, La Jolla, CA. The PCR product was purified and sequencing was performed with a panel of specific primers using the Sequenase kit as recommended by the manufacturer (US Biochemicals Corp., Cleveland, OH) exept that denaturation and annealing were performed by placing the reaction tube for 5 min at 95°C, then 5 min on ice and finally 20 min at room temperature. Cell clones with single integrations of the vector SL3-3-neo (16) with a slightly different enhancer region from Akv-neo (27) were included in parallel with all sets of reactions to verify the specificity of the method.

RNA dot-blot and Northern blot analysis

For RNA-dot blots, crude RNA was transferred to Zeta-probe membranes (Bio-Rad Laboratories, Richmond, CA) using a minifold essentially as described by Sambrook *et al.*, (28). The probe for detection of neo-RNA was a 1.8 kb DNA fragment encompassing the complete neomycin phosphotransferase II encoding region (29). The probe used for detection of actin-RNA used as an internal standard was a 1.1 kb murine gamma actin fragment. The probe used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was a 1.3-kb *PstI* fragment derived from pRGAPDH-13 (30). The probe used for chloramphenicol acetyl transferase (CAT) mRNA was a 0.3-kb *EcoRI–NcoI* fragment derived from pL6-cat (21). The DNA probes were ^{32}P labelled to a specific activity of about 5×10^8 dpm/μg essentially as described by Feinberg and Vogelstein (31). Hybridization and washing were as described in the Zeta-probe manual. Northern blot analysis was done by electrophoresis of 10 μg total RNA through 1.2% formaldehyde/agarose denaturation gels and transfer to Zeta-probe membranes in 10×SSC (1.5M NaCl, 0.15M sodium citrate, pH 7.0). Hybridization and labelling of radioactive DNA probes were as described above.

Quantification of hybridization signals

Dot-blot hybridizations were quantitated densitometrically on an ELISA reader (405 nm) using the absorption of the film outside the dots as blank value. This allows a more accurate quantitation than scintillation counting of the hybridized probe (32, 33). Only exposures in the range of proportionality between radioactivitiy and film density as determined by standard curves were used. Multiple measurements were performed on each dot and darker spots resulting from contamination were avoided. Nothern autoradiograms were scanned using a Shimadzu dual wavelength TLC-scanner at 590 nm.

RESULTS

Choice of model system

We wanted to determine the response to a regulatory signal of identical vector transcription units inserted at a set of random chromosomal positions. As a model we chose a set of cell clones of the murine lymphoid cell line L691 carrying random single integrations of a retroviral vector, Akv-neo (Fig. 1.), containing the *neo* gene driven by the promotor–enhancer region of the Akv murine leukemia virus long terminal repeat (LTR) and having a wide range of *neo* gene expression levels (16). For some of the clones the vector transcript was analyzed by RNase

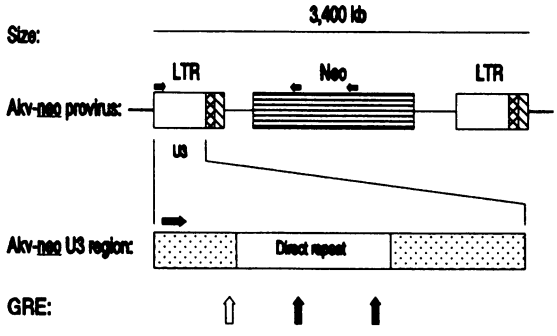

Figure 1. Schematic representation of the integrated vector provirus Akv-neo. Box with horizontal lines, Tn5 sequences including the *neo* gene; doubly crosshatched boxes, R region of the LTR; singly crosshatched boxes, U5 region of the LTR; open boxes, U3 region of the Akv-neo provirus; thin line, virus sequences; thick lines, host DNA flanking the integration sites of the vector proviruses; horizontal arrows, oligonucleotides used for PCR amplification of the integrated vector provirus; crosshatched vertical arrows, glucocorticoid response elements (GRE); open vertical arrow, putative GRE. Figures are drawn to scale.

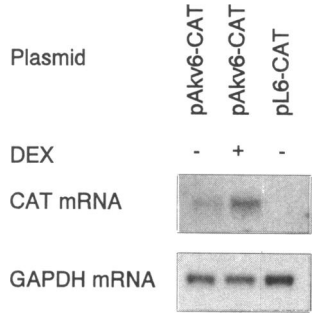

Figure 2. Transient expression analysis of L691 cells electroporated with pAkv6-cat and the negative control pL6-cat. After electroporation cells were grown for 44 hours, split in two and one half induced with 1 μM dexamethasone for 4 hours. RNA was isolated and used for Northern-blotting. A probe for the CAT gene was used to measure the transcriptional activity of the vectors (upper panels) and a GAPDH probe for estimation of total mRNA levels (lower panels). For RNA from the pAkv6-6 recipient cells the peak ares determined by scanning were: In the absence of Dex, 3.0 for the CAT probe and 38.3 for the GAPDH probe; in the presence of dex, 13.6 for the CAT probe and 36.2 for the GAPDH probe. No peak was detected in the pL6-cat recipients. Fold induction of the Akv-cat RNA by Dex was calculated to 4.8 (the ratio between CAT/GAPDH values with and without dexamethasone).

protection and the use of proper initiation and polyadenylation sites confirmed (16).

Dexamethasone has been found to stimulate transient expression of genes directed by the Akv enhancer in various lymphoid cell lines (18, 19). This enhancement is mediated through an interaction between the hormone–receptor complex and the glucocorticoid response elements (GRE) in the enhancer. For the Akv U3 region two GRE's and one putative GRE have been described (18). The locations of the GRE sequences (5′-TGGGGACCATCTGTTCT-3′) and of the putative GRE sequence (5′-TGTTCTTGTTTTTCTGAGAACA-3′) are indicated in Fig. 1.

The Akv-neo containing cell clones were obtained by infection of L691 cells with helper virus free stocks of Akv-neo particles. Transduced cell clones were identified by Southern blotting after

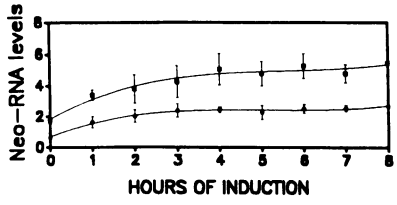

Figure 3. Dexamethasone induction kinetics for two single-integrated Akv-neo vector proviruses. Cells of clones A10 and A30 were induced with 1 μM dex for the indicated number of hours. Total RNA was isolated, applied to a hybridization membrane and hybridized with a *neo*-probe and the signal quantitated by densitometric analysis of autoradiograms in the linear range of exposure. The membrane was stripped of the *neo*-probe and rehybridized to an actin gene probe. The *neo*-expression levels were determined as a ratio between *neo* and actin signals (see Fig. 4) to correct for variations in the amount of RNA applied to the filters. The normalized *neo*-RNA levels are expressed in arbitrary units given relative to a standard *neo*-RNA sample common to all measurements. The curves show the average result of two independent induction experiments, each represented by hybridization analysis of two parallel RNA samples. The error bars give the standard deviation of these four measurements. Lower curve: A10; upper curve: A 30. Parallel dexamethasone induction experiments using non-transduced L691 cells gave no detectable *neo*-hybridization signals for all time points.

subcloning and end-point dilution without selection for *neo* gene expression (16). Expression levels might be affected by mutations introduced into the vector in the packaging cells, during vector replication or in the Akv-neo vector provirus during expansion of the cell cultures. For all cell clones we therefore amplified the enhancer–promoter region in the 5′ LTR of the vector by the polymerase chain reaction and determined selected nucleotide sequences of this region (U3). The positions of the oligonucleotide primers are shown in Fig. 1. Various specific sequencing primers allowed sequence analysis of the enhancer region (direct repeat) and the surrounding sequences. The region sequenced covered the two GRE's in the repeats as well as the upstream element with a potential role for the glucocorticoid response Fig. 1. No mutational differences were found in the sequenced region in any of the cell clones. Furthermore, no rearrangements could be detected by amplifying different parts of the vectors and subjecting the amplified products to agarose gel electrophoresis analysis (data not shown).

The effect of dexamethasone in L691 cells under transient expression conditions

Dex has been found to enhance transient expression of genes directed by the Akv enhancer in various lymphoid cell lines (19, 18). To measure the Dex effect in the L691 cell line on transient Akv LTR driven expression we used the vector pAkv6-cat and the homologous promoter–enhancer negative construct pL6-cat Fig. 2. DNA was introduced into L691 cells by electroporation and 44 h later the transfected cells were split in two aliquots that were then incubated either without or with Dex (1 μM). Dex inducibility was studied after an incubation time of 4 h (34). RNA was analyzed for the content of vector RNA transcripts by Northern blotting using a probe covering the CAT gene. To correct for variations in the amounts of RNA, the filter was stripped and rehybridized with a probe for the GAPDH gene as shown in Fig. 2. The intensities of the hybridization signals for the two probes were determined by densitometric scanning of autoradiograms and the CAT/GAPDH ratios used as normalized values for the level of CAT mRNA. In this manner a 4.8 fold increase between dex induced and uninduced levels of the

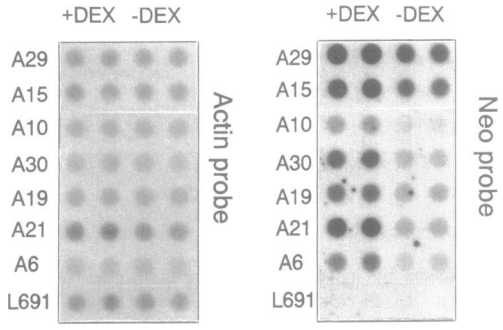

Figure 4. RNA dot-blot measurements of the effect of dexamethasone upon *neo*-expression in cell clones with single integrated vector proviruses. Total RNA was isolated using Eppendorf tubes, split into two samples and applied to a hybridization membrane. The filter was hybridized with a *neo*-probe and exposed to an X-ray film, upper panels. The membrane was stripped of the *neo*-probe and rehybridized to an actin gene probe, lower panels. In the examples shown here RNA was derived from seven Akv-neo clones (A29, A15, A10, A30, A19, A21 and A6) and the uninfected parental cell line L691.

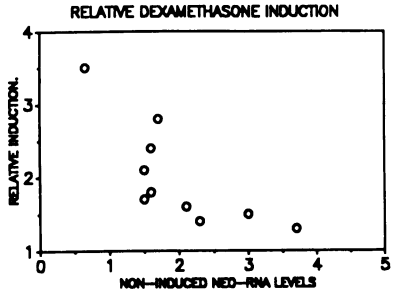

Figure 5. The relative induction levels for the individual Akv-neo clones as a function of their basal *neo*-RNA levels. Relative induction is defined as the ratio of the level of *neo*-RNA after dex addition to *neo*-RNA without dex. Relative induction and non-induced *neo*-RNA levels were calculated after standardization to actin hybridization signals as described in legends to Fig. 3. and Fig. 4. Each point is the average of two independent induction experiments where both experiments consisted of a double determination of both the induced and non-induced *neo*-RNA levels for all the Akv-neo cell clones. For reasons of transparency, error bars have been excluded. The accuracy was similar to that of the experiments reported in Fig. 3. In both experiments, RNA from uninfected L691 cells was included as negative control and the *neo*-hybridization signals found to be neglicable in all cases.

pAkv6-cat transcript was estimated. In an independent induction experiment the same ratio (4.8) was found using in this case poly (A) selected RNA for the Northern blotting analysis (data not shown). This ratio is similar to that previously observed for other lymphoid cell lines (19).

Dexamethasone induction of integrated vectors

Dex (1 μM) was added to cultures of two different cell clones (A10 and A30) and RNA was harvested before and at one hour intervals after induction. Vector encoded *neo* mRNA levels were determined by RNA dot-blot hybridization, followed by densitometry of autoradiograms using the actin mRNA level as an internal standard. As seen in Fig. 3. addition of dex caused an increase in the levels of *neo* mRNA within the first hour. A plateau was reached after about 4 h similar to what was observed for endogenous mouse mammary tumor virus *env* mRNA (35).

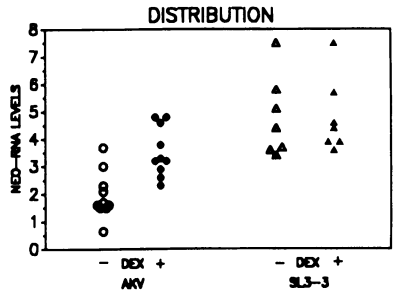

Figure 6. Distribution of *neo*-RNA levels from non-induced and induced Akv-neo vector proviruses and SL3-3-neo vector proviruses. The clone set of SL3-3-neo vector proviruses has previously been described (16). The *neo*-RNA levels were calculated as described in legends to Fig. 3. and Fig. 4. RNA levels are given relative to an arbitrary standard *neo*-RNA sample common to all measurements.

The effect of Dex addition upon vector expression was determined for all the Akv-neo cell clones using 4.5 h as a standard induction time. Repeated experiments were performed for each of the cell clones as described in the legend of Fig. 5. Examples of the RNA dot-blots used for these measurements are shown in Fig. 4. The relative induction values of vector expression were determined for all cell clones and plotted as a function of the non-induced RNA levels Fig. 5. The different proviral integrates showed a response to Dex, varying between about 3.5 fold and a value slightly above 1. The strongest relative inductions were observed for the vectors with the lowest uninduced expression levels (Fig. 5.).

A panel of random L691 cell clones with single integrated SL3-3-neo vector proviruses (16) was also included in these experiments. The SL3-3 promoter−enhancer structure carried by this vector is closely related to that of Akv. In contrast to Akv, the SL3-3 promotor-enhancer confers strong transient expression activity in lymphoid cells including L691 and shows no significant transient response to Dex in this cell line, although it carries GRE elements that are functioning in other cells (19, 18). We found little or no Dex inducibility of the vectors in these cell clones (Fig. 6.).

DISCUSSION

To determine how integration into various chromosomal sites affects the response of a transcription unit to a regulatory signal we studied the Dex response of the Akv promoter−enhancer, driving expression of the *neo* gene in a retroviral vector. The Dex inducibility of ten stably integrated single-copy vectors at random sites was determined in cell clones of the lymphoid line L691 where the weak Akv promoter−enhancer is induced several fold under transient expression conditions. The cell clones presumably represent the full range of vector expression levels since they were isolated at high cloning efficiencies without selection for vector expression (16).

The Dex stimulation of expression of the Akv-neo vector proviruses varied between 1.3 and 3.5 fold decreasing with increased uninduced expression levels. It is unlikely that the low stimulation of the higher expressed integrated vectors reflects a limited polymerase loading capacity of the transcriptional promoter, since some single integrates of vectors with the same promoter linked to the closely related SL3-3 enhancer were found to result in even higher expression levels (15). The 3.5 fold

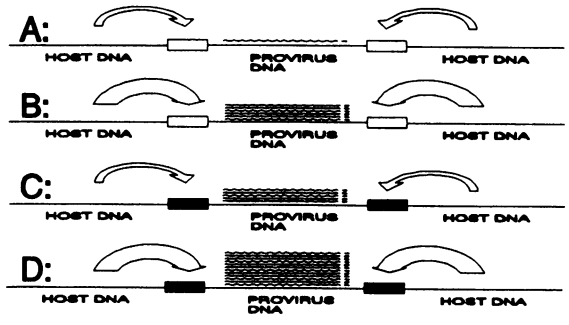

Figure 7. Schematic representation of vector enhancer strength and chromosomal position effects. The model considers only positive influence from chromosomal sites. The thin arrows (A and C) represent weak sites and the thick arrows (B and D) strongly stimulating sites. The model shows vector proviruses with weak enhancer activity (open box, e.g. Akv in L691 cells) or with strong enhancer activity (filled box, e.g. SL3-3 in L691 cells or Akv in L691 cells in the presence of dexamethasone). RNA expression of the vector proviruses is indicated by wavy lines. The model implies that expression of a vector with a weak enhancer is strongly affected by the integration site, whereas expression of a vector with a strong enhancer is only affected to a minor extent. Negative effects of integration may also occur and lead to complete shut-down of vector expression (see text).

stimulation of expression of the low-expressors among the integrated Akv-neo vectors approaches the induction observed in transient expression (4.8 fold), suggesting that the response of the lower expressors more clearly reveals the inherent regulatory pattern of an inserted transcription unit. This notion is of potential importance for studies of gene regulation after stable gene transfer, if recipient cells are selected for expression of the transferred transcription unit prior to further analysis. As was observed in case of the model system used for the present study (15), selection may eliminate the low-expressors in a population of recipient cells, and as we have demonstrated here thereby also eliminate those cells in which the transferred transcription unit most clearly follows its inherent regulatory mechanisms.

The Dex response of the Akv LTR has been assigned to GRE motifs in the enhancer repeat region of U3 with a possible additional role of a related upstream U3 sequence (18). The DNA-sequence of the region containing these elements is intact in the vectors of all cell clones, and the observed inducibility pattern most likely results from a stimulation through the vector GRE's in combination with an influence from the integration site. Hormone responsive elements in host DNA close to the integration sites might theoretically also contribute to the Dex response of the vectors. Such effects are, however, most likely of only minor importance for the observed differences in inducibility, because of the correlation with basal expression levels. After Dex addition, the distribution of expression of the integrated Akv-neo vectors approaches that observed for the SL3-3-neo vectors (Fig. 6.), where transcription is driven by the SL3-3 murine leukemia virus enhancer that, as determined in transient expression assays, is about an order of magnitude stronger than the Akv enhancer in L691 cells (16, 36, 37, 38). The lack of inducibility of the integrated SL3-3-neo vectors indirectly supports the notion that the dex response of the Akv-neo vectors operates through vector sequences since the SL3-3 promoter–enhancer shows little or no Dex response in lympohid cells in transient expression assays (19, data not shown). Dex may therefore be considered as altering the enhancer strength of the integrated Akv-neo vectors *in situ* to that of a stronger enhancer.

The model in Fig. 7. (16, 39) illustrates positive chromosomal position effects on vector expression. At the non-stimulating or weakly stimulating sites the expression of the vectors is largely dependent upon the inherent enhancer strength of the vectors (Fig. 7. A and C), resulting in the large relative difference between the low-expressors of the Akv-neo and SL3-3-neo clone sets. At a strongly stimulating chromosomal site, on the other hand, the relative difference between expression of vectors with a weak and with a strong enhancer is minor (Fig. 7. B and D). Looking at Dex inducibility of the integrated Akv-neo vectors, we observe the strongest relative effect for the low-expressors, where the inherent enhancer strength, and not the chromosomal position, is the major determinant of expression level. For the stronger expressing Akv-neo clones, the integration site may contribute more to expression than does the inherent enhancer strength, and since only the enhancer strength is affected by Dex, a smaller relative effect is observed.

We previously observed efficient replication of mutant murine retroviruses with strongly reduced transcriptional enhancer strength both in cultured cells (40) and in target cells in infected animals (41). Stimulatory effects on expression of integrated vectors with weak enhancers from some sites of integration as proposed in Fig. 7. may provide an explanation for these observations.

Work in *Drosophila melanogaster* has identified position effects of positive or negative (silencer) enhancer elements as well as negative effects causing inactivation of expression related to chromatin structure (1). Using this conceptual framework the effects observed here resemble the first type caused by transcriptional enhancers at the integration sites. In studies of positive and negative effects of the chromosomal positions on stable expression levels of transferred minigenes in *Drosophila melanogaster*, the negative effects were most readily observed on strong transcriptional elements whereas the positive effects were best detected on weak transcriptional elements (42). In our work the use of the relatively weak promoter–enhancer region of Akv in L691 cells and the lack of any selection step during isolation of infected cell clones have allowed the detection and analysis of strong, positive position effects. No clear indication of position effects resulting from negative enhancers (silencers) emerged from our studies, not even when the stronger SL3-3 enhancer was used (Fig. 6).

Negative effects on transcription in mammalian cells may be associated with gradual inactivation as a function of time. Such effects often occur in combination with DNA methylation and may be analogous to the chromatin-structure related effects of *Drosophila melanogaster*. A number of such cases have been described for retroviral vector proviruses (43, 44, 45, 46). The effects of integration site on Dex inducibility and vector inactivation described for mouse mammary tumor viruses (47, 48) most likely result from this kind of negative effect that may be associated with DNA-methylation (49). Inactivating effects of this type have also been observed during long-term cultivation of some but not all of the cell clones used in the present study (16, 50). Negative position effects of this kind superimpose in a dominant manner upon the positive effects that are presented here and cannot be counteracted by a short Dex treatment, in contrast to the stable low-expressors among the integrated vectors that show a strong relative response as described here. This distinction is compatible with the central proposal of the present

communication, that the lower expression levels result from a lack of positive position effects rather than from dominant negative effects.

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