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Enhancer Functions in U3 of Akv Virus: a Role for Cooperativity of a Tandem Repeat Unit and Its Flanking DNA Sequences

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***cis*-Acting transcriptional control elements in the U3 region of the murine retrovirus Akv were analyzed in mouse NIH 3T3 fibroblast cells by using a transient expression vector system based upon a complete long terminal repeat with linked flanking sequences. Deletion analysis pointed to the essential role of sequences within the 99-base-pair direct repeats, and a fragment encompassing the two repeats was found to possess orientation-independent enhancer activity when positioned either upstream or downstream of the transcriptional unit. Removal of one copy of the 99-base-pair repeat led to a reduction in activity of about 2.5-fold when located in an intact U3 environment but to reductions of up to 2 orders of magnitude when placed in other sequence contexts. Our studies of enhancer functions in the presence of one versus two copies of the tandem repeat point to duplicate functions of repeat sequences and sequences flanking the repeat region and emphasize the complex overall organization of this U3 region.**

Tandemly repeated sequences are found in the U3 regions of many retroviruses and in transcriptional control regions of other animal viruses. In the murine leukemia viruses Akv (11, 20, 42), SL3-2 (8a, 33), SL3-3 (25), Gross passage A (44), FBJ (24, 43), OA-I (24), FBR (41), Soule (8), B16-5 (10), and related endogenous viruses (6, 7, 42), the repeat regions are characterized by rearrangements, duplications, or triplications involving a small number of sequence modules, while the remaining U3 sequences are almost identical. Other murine retroviruses, such as the Moloney murine leukemia virus and Moloney murine sarcoma virus (MoMSV) (36), harbor repeat regions composed of sequence modules very similar to those found in the Akv group of viruses, while their sequences outside the repeat region show less stringent homology to Akv. However, the distance from the repeat region to the cap site is identical in all these viruses (M. Etzerodt, Ph.D. thesis, University of Aarhus, Aarhus, Denmark, 1987).

While many reports have pointed to the crucial role of the repeat region in transcriptional enhancement, little is understood about the role of the overall organization of the U3 regions of the murine leukemia viruses. The wide interest in the transcriptional control and oncogenicity of these viruses led us to undertake a detailed study of transcriptional functions in the U3 region of a prototype virus, Akv murine leukemia virus, carrying a 99-base-pair (bp) tandem repeat.

A vector system based upon a complete Akv long terminal repeat (LTR) with the linked 5′-untranslated sequences of the virus was used for our studies of the effect of deletions and/or rearrangements of U3 sequences upon transient expression in murine fibroblasts. We confirmed the critical role of sequences within the tandem repeats for enhancer functions (2, 4, 5, 8a, 15, 17, 23, 29, 37), but, unexpectedly, sequences in U3 outside the repeats were found to have a marked effect in constructs with only one copy of the 99-bp sequence. Our results therefore reveal new aspects of the organization of murine leukemia virus U3 regions and stress the importance of studying elements of these in their natural sequence environment.

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MATERIALS AND METHODS

Cells. The NIH 3T3 mouse fibroblast cell line was grown in Dulbecco modified Eagle medium–10% newborn calf serum–100 U of penicillin per ml–100 μg of streptomycin per ml–4 mM L-glutamine (all components were obtained from Seromed). Cells were incubated at 37°C in 90% relative humidity and 5.7% CO_2.

Plasmids. The expression vector plasmids used in this work and in our previous studies (8a, 32) contain a transcription unit with initiation site and 5′-untranslated sequences from Akv, a chloramphenicol acetyltransferase (CAT) reporter gene, and a small intron and polyadenylation site of simian virus 40 (Fig. 1). The plasmid pAkv6cat contains a complete Akv U3 region. The plasmids of the pD series (pD1 through pD17) differ from pAkv6cat by random deletion of a single stretch of nucleotides in U3 or in U3 and upstream sequences (Fig. 1 and 2). The pD plasmids were generated by ligation of *Bal* 31-digested fragments from two mother plasmids carrying *amp* and *tet*, respectively, as previously described (33). By cleavage with *Apa*I and self-ligation, one copy of the 99-bp tandem repeat sequence was excised from pAkv6cat to generate plasmid p1-99 and from plasmids of the pD series to generate plasmids of the pDD series (pDD1 through pDD7 and pDD14) (Fig. 1 and 2).

The pAkv6cat derivatives pAkv8.1, pAkv8.2, and pAkv8.3 (Fig. 1, 2, and 3) contain chemically synthesized linker DNA with unique restriction sites allowing fragment insertion in *Xba*I and *Xho*I sites in a modified U3 or in a *Stu*I site 3′ of the polyadenylation site.

The plasmids pA1 through pA12 (Fig. 3) represent derivatives of pAkv8.1, pAkv8.2, and pAkv8.3 with an *Alu*I fragment of Akv U3 (bp −407 through −169) inserted in either orientation into polylinker sequences either upstream (in the *Xba*I site) or downstream (in the *Stu*I site) of the transcriptional unit. From these 12 plasmids, derivatives pA13 through pA24 (Fig. 3), with only one copy of the 99-bp repeat sequence, were subsequently generated by *Apa*I digestion followed by self-ligation.

Plasmids used for transfection were purified by equilibrium density gradient centrifugation in CsCl, and the nucleotide sequence of the critical regions was determined (18) by

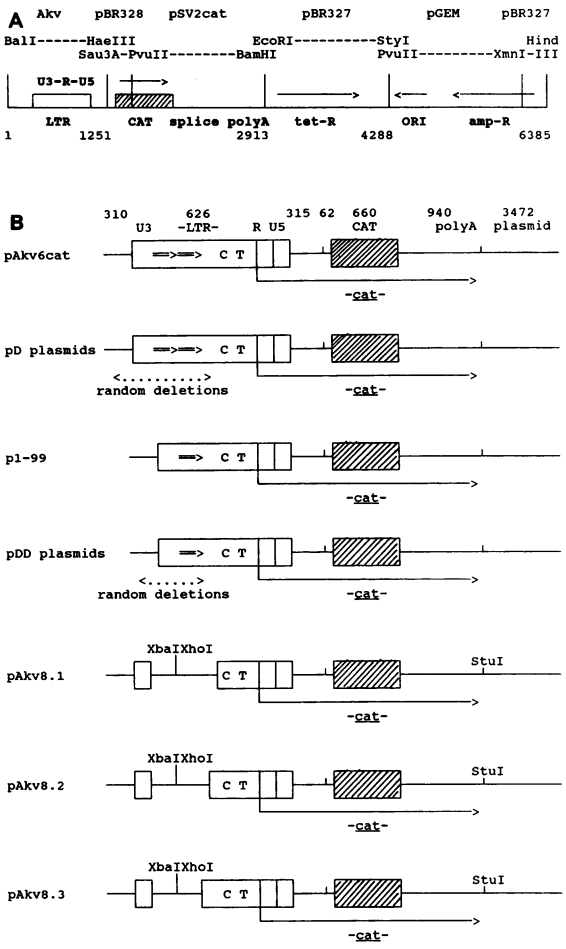

FIG. 1. Plasmids used in transient expression assays. (A) Origin of nucleotide sequences in the basic expression vector plasmid pAkv6cat. The complete plasmid is drawn in a linear form with the Akv-derived sequences at left. The pAkv6cat bp 1 through 6385 are numbered from left to right as shown. bp 1 through 1251 correspond to Akv bp −792 through +459 as numbered relative to the bp +1 at the U3-R border (pAKR59 nucleotides 7515 through 459 [11, 20, 26, 42]). bp 1252 through 1254 are linker sequences (GGG on the vector plus strand). bp 1255 through 1427 are from pBR328 (a *Sau*3A-*Pvu*II fragment encompassing the start of the CAT coding region; pBR328 bp 149 through 321 [38]). bp 1428 through 2913 were derived from pSV2cat (a *Pvu*II-*Bam*HI fragment containing the rest of the CAT coding region and simian virus 40 t-antigen intron and polyadenylation sequences; bp 149 through 1634 in pSV2cat [13]). bp 2914 through 4288 and 5957 through 6385 were derived from pBR327 (*Eco*RI-*Sty*I and *Xmn*I-*Hin*dIII fragments, respectively; pBR327 bp 3273 through 1373 and 2879 through 0033 [38]). bp 4289 through 5956 represent a *Pvu*I-*Xmn*I fragment of pGEM1 (Promega Biotec) (pGEM1 bp 99 through 1766). (B) Maps of pAkv6cat-derived plasmids. The pD plasmids contain a single deletion relative to pAkv6cat, p1-99 retains only one 99-bp repeat, and the pDD plasmids contain a single deletion with respect to p1-99. All three pAkv8 plasmids contain a 13-bp linker 5′-GGATCCCTTAGGCCTC CGAATTC-3′ inserted after bp 2913 between *Bam*HI and *Eco*RI sites in pAkv6cat. The U3 region of the three plasmids has been modified by insertion of a 19-bp polylinker (Fig. 2) replacing the *Pst*I-*Dra*I fragment (bp 352 through 704) in pAkv8.1, the *Pst*I-*Sau*3A fragment (bp 352 through 683) in pAkv8.2, and the *Pst*I-*Ava*II fragment (bp 352 through 608) in pAkv8.3. The 99-bp base pair repeats in U3 are indicated (⇒), and C and T refer to the CCAAT and TATA boxes, respectively. The transcriptional unit containing R, U5, and untranslated sequences from Akv, CAT-coding sequences, and simian virus 40 splicing and polyadenylation signals is represented by *-cat-*. *Pst*I and *Kpn*I are unique sites in all plasmids. *Apa*I is unique to the 99-bp repeat. *Xba*I, *Xho*I, and *Stu*I sites in the linkers are unique in the corresponding vectors.

using a panel of specifically synthesized oligonucleotide primers.

The two β-galactosidase expression vector plasmids pCH110 (16) and pGA293 (1) were used as internal standards to correct transient expression measurements for variations in transfection efficiencies.

Transfections and expression assays. Transfections were performed by the calcium phosphate precipitation method (14) by using a glycerol shock (28) with 5 μg of reporter plasmid DNA, 6 or 8 μg of internal standard plasmid DNA, and 4 μg of salmon sperm DNA to 10^6 cells. For mock transfections, reporter plasmid DNA was replaced by 5 μg of salmon sperm DNA. After incubation for 46 h, the cells were harvested and lysed by freeze-thaw cycles. The extract was cleared from cell debris by centrifugation, and the supernatant was used for determination of CAT activity (13) and β-galactosidase activity (19). CAT activity was determined in 1- or 2-h incubations with an amount of extract that ensured substrate conversion in the linear range, normalized to the β-galactosidase activity, and corrected by subtraction of mock values. CAT activity is given relative to the mean value for the parallel pAkv6cat plasmid transfections, which was arbitrarily set at 100. The methods allowed determinations of CAT activities in a linear range over about 3 orders of magnitude. For each plasmid construct, 6 to 26 independent transfections were performed.

RESULTS

Expression vector plasmids. Our standard expression vector plasmid pAkv6cat (Fig. 1) carries a 1.25-kbp Akv fragment with a complete LTR and adjacent sequences from the 3′ and 5′ ends of the viral genome linked to a CAT reporter gene and a simian virus 40 polyadenylation site as shown in Fig. 1. The expression vector has been found to use the correct transcriptional initiation site (data not shown).

The plasmids of the pD series (pD1 through pD17) (Fig. 1 and 2) are pAkv6cat derivatives with a deletion of a single stretch of nucleotides in U3 and adjacent sequences. By further excision of one copy of the 99-bp repeat, a series of double-deletion plasmids (pDD series; pDD1 through pDD7 and pDD14) was generated from the corresponding plasmids of the pD series (Fig. 1). Similarly, plasmid p1-99 was derived from pAkv6cat by excision of one 99-bp unit (Fig. 1). The plasmids pAkv8.1, pAkv8.2, and pAkv8.3 (Fig. 1) are pAkv6cat derivatives with replacement of U3 sequences by DNA with multiple restriction sites and with additional restriction sites introduced downstream of the polyadenylation site.

Effect of 5′ deletions in U3. To study the effect of 5′ deletions in U3, the 21 plasmids (pD1 through pD13; pDD3 through pDD7; and pAkv8.1, pAkv8.2, and pAkv8.3) (Fig. 2) with deletions that start at or upstream of the *Pst*I site at bp −441 were used. It should be noted that the plasmids pAkv8.1, pAkv8.2, and pAkv8.3 carry a short linker sequence at the site of deletion (Fig. 2). Transient expression levels were determined by measurement of CAT activity after transfection into NIH 3T3 mouse fibroblasts, and the activities were plotted on a logarithmic scale versus the downstream deletion borders (Fig. 4). With two exceptions,

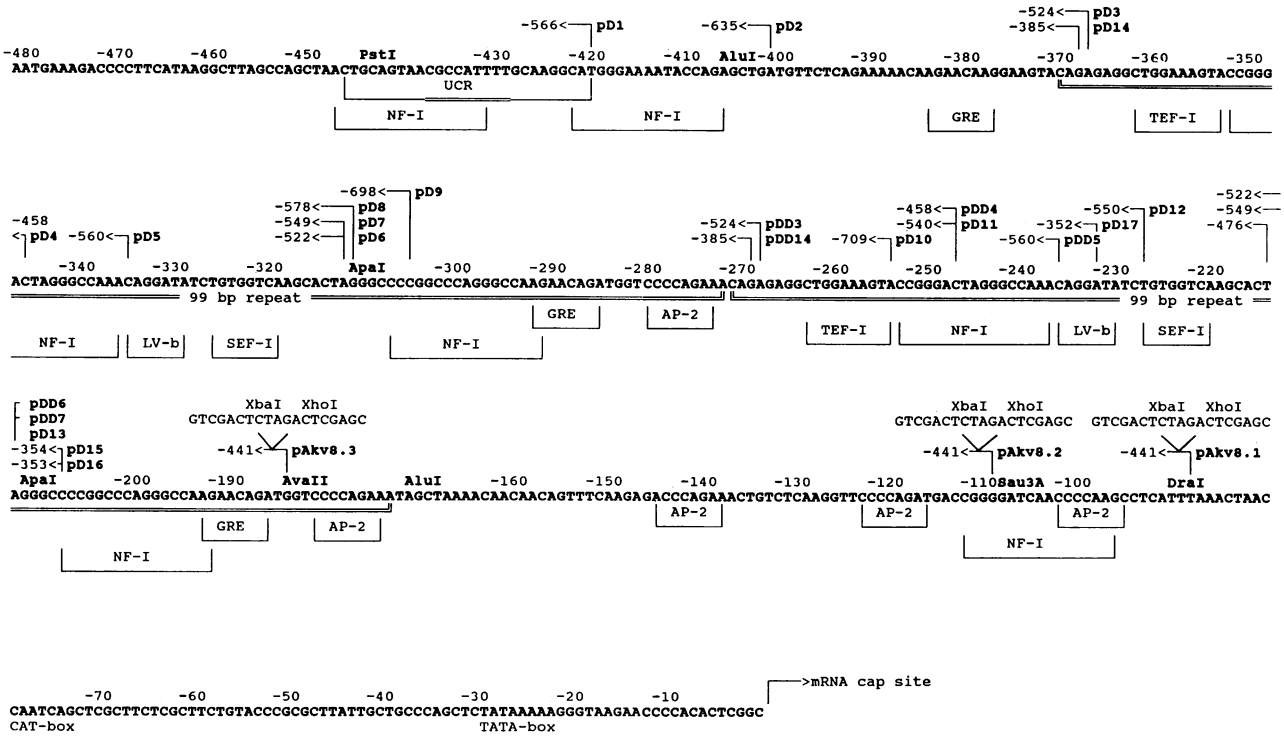

FIG. 2. Map of deletions in the U3 region of Akv. The positions in the nucleotide sequence of the U3 region of the long terminal repeat of Akv (11, 20, 26, 42) are numbered relative to the mRNA cap site at the U3-R border. The 99-bp direct repeats and CCAAT and TATA boxes are indicated below the sequence together with the following DNA motifs with a possible role in regulation of transcription: UCR (12), NF-I (22, 27; Olsen et al., submitted), GRE (5, 30, 34), TEF-I (9), LV-b (39), SEF-I (40), and AP-2 (21, 31, 45). The deletion borders of the deletion mutants (pD1 through pD18, pDD1 through pDD7, and pDD14) and the three pAkv8 vectors (pAkv8.1, pAkv8.2, and pAkv8.3) are indicated above the sequence. The arrows with negative numbers indicate the first and last base pairs of the deleted sequences. In the pAkv8 vectors, the deleted sequence is replaced by a polylinker sequence as shown.

pD7 and pD13, the deletions showed either unaltered or gradually declining activity upon successive removal of U3 sequences. We infer that the activity of deleted plasmids is primarily determined by the remaining U3 sequences, irrespective of the variation in upstream borders of the deletions.

Sequences upstream of the tandem repeats can be deleted without any effect upon transient expression, while further deletions cause a decline in activity amounting to a fourfold reduction upon complete removal of the distal repeat (Fig. 4). Deletions into the proximal repeat led to marked reductions in activity, the most dramatic effect being observed for bp −268 to −255 and for bp −254 to −248, possibly representing binding sites for transcription factors homologous to transcription enhancer factor 1 (TEF-I) (9) and nuclear factor I (NF-I) (3, 22; Olsen et al., submitted for publication) (Fig. 2). Deletions eliminating the proximal repeat and downstream sequences caused a sequential loss of activity, with a drastic effect resulting from removal of sequences upstream of the CCAAT box.

Effect of the presence of one or two 99-bp repeats. To study the role of the tandem duplication, one copy of the 99-bp sequence was excised from pAkv6cat and from four plasmids (pD1, pD2, pD3, and pD14) harboring U3 deletions upstream of the repeats. Removal of one tandem repeat (Fig. 3A) caused a reduction in expression activity to about 40% for pAkv6cat, as it did for pD14 harboring a deletion just upstream of the tandem repeat sequence from bp −385 to −369. The removal of one copy of the tandem repeat from the plasmids pD1, pD2, and pD3, however, had a more pronounced effect, causing about a fourfold reduction in expression.

Whereas the deletion series in Fig. 4 showed that removal of sequences upstream of the two repeats had no effect, these sequences seemed to play a role when only one copy of the 99-bp repeat unit was present. It was therefore of interest to test whether these sequences might activate transcription in the absence of 99-bp repeat elements. Three deletion mutants of pAkv6cat (pD15, pD16, and pD17) (Fig. 3B), affected exclusively in tandem repeat sequences, showed activities about 50-fold lower than that of pAkv6cat. These activities were in the same range as those found for plasmids with both the tandem repeat and 5′ sequences deleted. An effect of the sequences upstream of the tandem repeats could therefore only be demonstrated in the presence of one 99-bp repeat unit.

One versus two repeats in foreign sequence contexts. To further study the function of the 99-bp repeat sequences and their interaction with surrounding DNA, the vector plasmids pAkv8.1, pAkv8.2, and pAkv8.3 (Fig. 1 and 3C) were used for insertion of fragments at unique restriction sites either in the U3 region or downstream of the polyadenylation site.

In the first series of derivatives (pA1 through pA6) (Fig. 3D), the *Alu*I fragment (bp −407 through −169) encompassing both tandem repeats was inserted in either orientation at the *Xba*I site in the modified U3 region of pAkv8.1, pAkv8.2, or pAkv8.3. All six derivatives showed expression levels similar to or higher than that of pAkv6cat. Removal of one copy of the 99-bp repeat sequence from plasmids pA1 through pA6 caused a dramatic reduction in activity (pA13

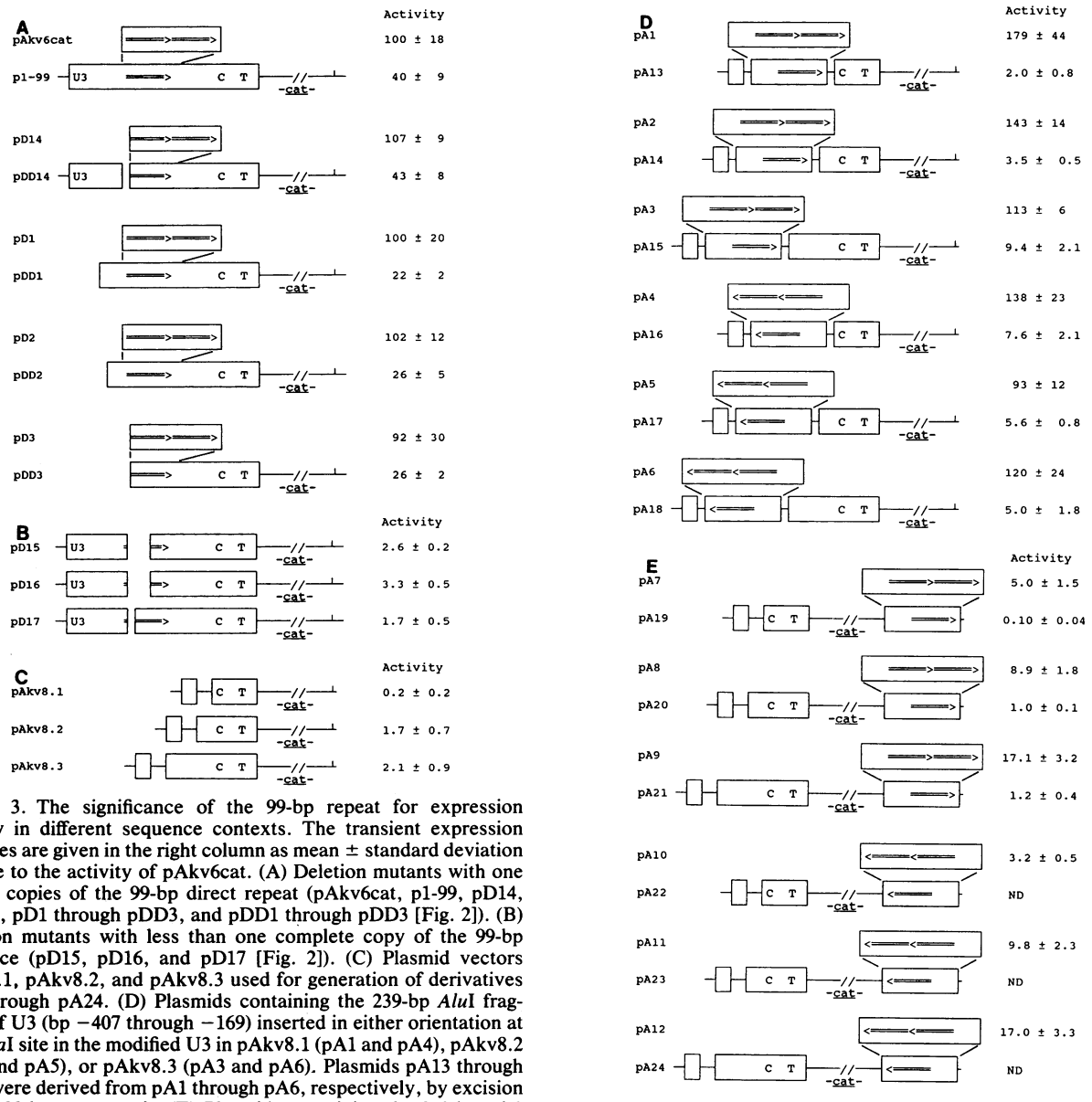

FIG. 3. The significance of the 99-bp repeat for expression activity in different sequence contexts. The transient expression activities are given in the right column as mean ± standard deviation relative to the activity of pAkv6cat. (A) Deletion mutants with one or two copies of the 99-bp direct repeat (pAkv6cat, p1-99, pD14, pDD14, pD1 through pDD3, and pDD1 through pDD3 [Fig. 2]). (B) Deletion mutants with less than one complete copy of the 99-bp sequence (pD15, pD16, and pD17 [Fig. 2]). (C) Plasmid vectors pAkv8.1, pAkv8.2, and pAkv8.3 used for generation of derivatives pA1 through pA24. (D) Plasmids containing the 239-bp *Alu*I fragment of U3 (bp −407 through −169) inserted in either orientation at the *Xba*I site in the modified U3 in pAkv8.1 (pA1 and pA4), pAkv8.2 (pA2 and pA5), or pAkv8.3 (pA3 and pA6). Plasmids pA13 through pA18 were derived from pA1 through pA6, respectively, by excision of one 99-bp repeat unit. (E) Plasmids containing the 239-bp *Alu*I fragment of U3 (bp −407 through −169) inserted in either orientation at the *Stu*I site downstream of the polyadenylation site in pAkv8.1 (pA7 and pA10), pAkv8.2 (pA8 and pA11), or pAkv8.3 (pA9 and pA12). Plasmids pA19 through pA24 were derived from pA7 through pA12, respectively, by excision of one 99-bp base pair unit. Symbols correspond to those of Fig. 1. ND, Not determined.

through pA18) (Fig. 3D). For pairs of homologous plasmids, the activity ratio between constructs with two and one repeat units was in the range of 10 to 100. One repeat unit therefore functions much better in its natural sequence environment (p1-99) (Fig. 4A) than in the pAkv8 vectors, in which sequences both 5′ and 3′ to the inserted repeat unit have been manipulated. Together with the effect of 5′ deletions described above, these results indicate a role for sequences both upstream and downstream of the 99-base-pair sequence. We cannot exclude, however, that our results are also affected by changes in critical distances in the promoter-enhancer region and especially that manipulations downstream of the repeat sequence may be critical in this respect.

To further analyze such position effects, we inserted the *Alu*I fragment containing the tandem repeat sequences in either orientation at a 2.2-kbp distance from the promoter at the *Stu*I site downstream of the polyadenylation site in the three pAkv8 vectors. The six resulting plasmids (pA7 through pA12) all gave expression values (Fig. 3E) above those obtained for the corresponding vectors without insert (Fig. 3C). The values differed for the three types of vectors but seemed to be independent of the orientation of the inserted fragment. All six plasmids yielded expression values lower than those of pAkv6cat and the plasmids with the *Alu*I fragment inserted in the upstream position (Fig. 3D). To study the effect of the removal of one 99-base-pair repeat in the downstream position, derivatives (pA19 through pA24, Fig. 4E) carrying only one repeat unit were generated. Their expression activities were found to be indistinguishable from those obtained for the vectors without inserted fragments,

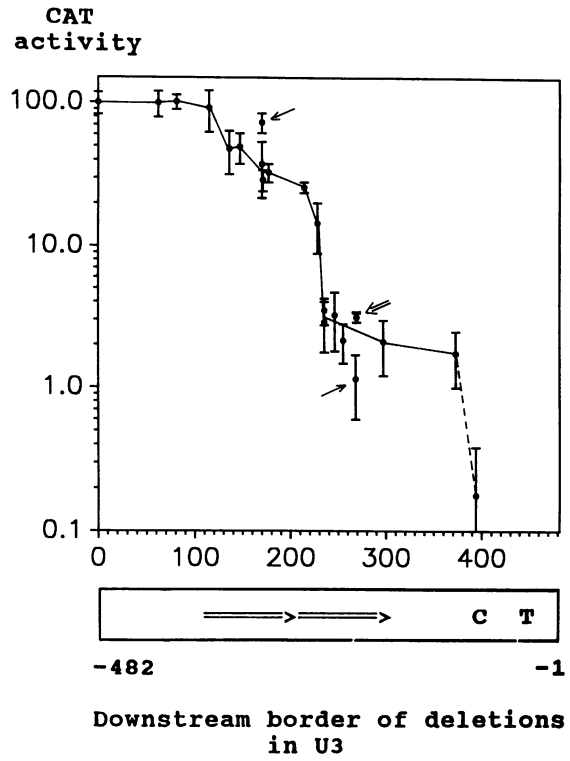

FIG. 4. Effect of upstream deletions in U3 on expression in NIH 3T3 fibroblasts. The expression activity (CAT activity) of 21 plasmids with deletions in the U3 region is plotted versus the downstream deletion border (corresponding to the arrows in Fig. 2) with a logarithmic scale. The mean expression activity and standard deviation (bars) of each plasmid are indicated. Plasmids represented are (from left to right and top to bottom): pAkv6cat, pD1, pD2, pD3, pD4, pD5, pD7, pD6, pD8, pD9, pDD3, pD10, pD11, pDD4, pDD5, pD12, pDD6, pDD7, pD13, pAkv8.3, pAkv8.2, and pAkv8.1. The two plasmids pDD6 and pDD7 are represented by a single graph point (⇒), since they have the same downstream deletion border and yielded indistinguishable activities. The curve has been drawn by hand. Since the value for pAkv8.1 was close to the sensitivity limit of the assay, the last part of the curve is indicated by a broken line. Two deletion mutants, pD7 and pD13, (marked by arrows) diverged significantly from the curve. The box below the graph represents U3 sequences, the arrows represent the 99-bp direct repeats, and C and T represent the CCAAT and TATA boxes, respectively.

i.e., at least 1 order of magnitude lower than the values obtained in the presence of both 99-bp repeats. The marked effect of the presence of one versus two copies of the repeat was therefore observed for insertion in upstream as well as downstream positions. We conclude that a single copy of the repeat sequence functions best when located in its natural sequence context, whereas two repeats in tandem function more independently of sequence environment.

Upstream promoter elements. The expression activities of the three vectors pAkv8.1, pAkv8.2, and pAkv8.3 suggested a role for sequences between bp −184 and −89 (Fig. 3E and 4). This is supported by the difference between the activities of the same three vectors containing the *Alu*I fragment inserted downstream of the polyadenylation site (pA7 through pA12) (Fig. 3E).

With the plasmids pA1 through pA6 containing the *Alu*I fragment inserted in the upstream position, high activities were observed even in the absence of bp −184 through −89. These sequences are therefore not absolutely required for activity. They may contain protein-binding sites that are implicated in the function of upstream promoter elements and, by deletion, such sites may be functionally replaced by other protein-binding sequences located in the 99-bp sequences, thereby explaining the high activity of pA1. If, however, no protein-binding sites are present in the sequences juxtaposed to the promoter region, a less active transcription complex will be formed, even in the presence of a strong enhancer, as in pA7.

DISCUSSION

We have studied the effects of deletions or rearrangements of U3 sequences upon Akv LTR-driven expression in mouse NIH 3T3 fibroblast cells. Bearing in mind the possible effect of novel junction sequences or of alterations in specific distances within the vector plasmid, we have been cautious to base our general conclusions upon results with multiple constructs.

The essential role of the 99-bp repeat region clearly emerges from our 5′ deletion analysis and is further supported by the effect of internal deletions in the repeat region. Removal of one repeat copy caused a 2.5-fold reduction in activity, and further removal of sequences in the remaining repeat reduced the activity an additional 10- to 20-fold. The importance of the repeat region for expression in fibroblast cells is in accordance with previous deletion studies of the MoMSV U3 region (15, 23) carrying two 75-bp repeats. The repeat sequences also play a major role for expression from the Akv-related SL3-3 (2, 17, 29) and SL3-2 (8a) U3 regions that both show strong expression in T-lymphoma cells. Within the repeats, we note the importance of sequences with homology to binding sites for transcription factors NF-I and TEF-I. The role of the NF-I motif is also supported by the low activity of our repeat internal deletion mutants, especially pD17. Transient expression assays using p1-99 derivatives with specific point mutations in the NF-I motif (Olsen et al., submitted) further support this interpretation. This crucial role of the NF-I motif, however, was not detected in the 75-bp repeats of MoMSV in a heterologous promoter assay (35).

The importance of sequences upstream of the CCAAT box is evident from the 5′ deletion analysis and is further supported by the results for plasmids (pA7 through pA12) with the repeat region in a 3′ position. This effect of upstream promoter deletions was, however, not observed when the repeat region was positioned 5′ of the promoter (pA1 through pA6), most likely because of functional replacement of protein-binding sites in the upstream promoter by other protein-binding sequences in the 99-bp repeat. Similar mechanisms may explain the apparent lack of a positive effect of MoMSV upstream promoter sequences upon expression in fibroblasts in the 3′ deletion series of Graves et al. (15).

In previous studies of enhancer function (5, 46), it was found that fragments containing the two 99-bp repeats of the Akv U3 region were active in either orientation when assayed at the 5′ position of a heterologous promoter. In this study, we have shown that the *Alu*I fragment encompassing the tandem repeats of Akv U3 possesses general enhancer activity when positioned in either orientation at both 5′ and 3′ locations with respect to the transcriptional unit, with the strongest stimulatory effects resulting from the 5′ insertions. Proximity to the promoter may determine this 5′ position preference, or the *Alu*I fragment may not represent the full

Akv enhancer, with additional U3 sequences contributing to its activity.

Our results provide new insight into the enhancer function of one versus two copies of the tandem repeat sequences. We first noted the deletion of sequences 5′ of the repeat sequence had no effect when two repeats were present (pD1, pD2, and pD3), whereas a significant twofold reduction was observed when only one repeat copy was present (pDD1, pDD2, and pDD3). This result points to duplicate functions of repeat sequences and sequences upstream of the repeat region. We subsequently detected even more dramatic differences of 10- to 100-fold between isogenic plasmids with one or two tandem repeats, as shown by the plasmid series pA1 through pA24, in which sequences both upstream and downstream of the repeat region were manipulated. Sequences on both sides of the repeat region therefore seem to play a role for full activity when only one copy of the repeat sequence is present. These effects were also observed when the repeat sequences were placed downstream of the transcription unit and therefore can hardly be explained solely by unfavorable distances between promoter elements and control elements in the 99-bp sequence for the single-repeat plasmids.

Related experiments have been reported for the MoMSV LTR by Laimins et al. (23). Their results, obtained by using the heterologous simian virus 40 promoter, disagree with ours. A single repeat unit from the MoMSV U3 was found to possess an enhancer activity the same as that of two tandem repeats when inserted in both orientations either 5′ or 3′ of the transcriptional unit. This discrepancy with our work is most likely due to the use of different promoters, since Laimins et al. (23), although they did not directly address the issue of promoter effects, also reported that a single MoMSV repeat unit did not stimulate the activity of its homologous promoter. Detailed inspection of the homologous promoter data presented by Laimins et al. (23) revealed a complete analogy with our results with respect to the effect of deletions upstream of a single MoMSV repeat unit. Furthermore, we have shown a similar interplay between repeat and upstream sequences for the SL3-2 virus (8a). We therefore believe that the cooperativity between a single repeat unit and its flanking DNA is of general relevance for the function of U3 regions of this family of viruses.

Because of their difference in dependence upon flanking sequences, U3 regions with one or two repeats should be treated as distinct control units which differ not only in the strength of their enhancers but also in their functional organization. Different relative effects of deletion of one repeat copy in different cell types, as have been observed for some viruses (2, 8a, 17, 29), may support this notion.

In summary, our analysis has confirmed the importance of repeat sequences, pointed to a cooperative role for sequences upstream of the repeats, and assigned upstream promoter functions and, probably, cooperative enhancer functions to sequences between the repeats and the CCAAT box. The significance of sequences outside the repeat region is not evident from a simple deletion analysis in the presence of two repeat copies, as demonstrated by our studies and by previous results (15). In the presence of only one repeat copy, however, the role of sequences outside the repeats could be demonstrated. Tandem duplications may therefore not only lead to increased strength of the transcriptional control region but may also reduce the importance of other U3 sequences, thereby permitting more frequent mutations outside the repeats during virus replication. In conjunction with successive tandem duplications and deletions of repeat copies, such mutations may therefore play an important role in viral evolution.

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