Tissue specific sequence motifs in the enhancer of the leukaemogenic mouse retrovirus SL3-3

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ABSTRACT

The long terminal repeat (LTR) of the retrovirus SL3-3 determines its tropism for T-lymphocytes and its ability to induce T-cell lymphomas in mice. We have studied the ability of different DNA sequences located upstream of the "TATA" box in the LTR of SL3-3 to enhance transcription in T-lymphocyte cell lines and other cell lines, employing a transient assay and quantitative S1 nuclease mapping. The enhancer was found to be composed of many DNA domains which determines different activities in different cell lines. We find enhancer sequence motifs with a high T-lymphocyte specificity in the DNA repetitions of the LTR, and other enhancer motifs active in a broader range of cells in the surrounding DNA segments. The localization of sequences preferentially active in T-cells within the repeated sequences containing differences between SL3-3 and the very closely related Akv virus, which is without the T cell tropism and leukaemogenicity of SL3-3, supports the notion that the enhancer sequence motifs with T-cell preferences are primary determinants of these properties.

INTRODUCTION

Enhancers are cis-acting DNA segments which dramatically stimulate transcription from RNA polymerase class B (II) homologous and heterologous promoters. They act over long distances in an orientation independent manner when located either upstream or downstream from the RNA start site. A large number of both viral and cellular enhancers have been described. There are many short sequence homologies between enhancers, with different enhancers having various assortments of such sequence motifs which together build up the enhancing activity. Sequence motifs of enhancers have been shown to be binding sites for different sequence specific trans-acting factors. Enhancers exhibit quite different degrees of host cell preferences in their activity, ranging from the simian virus 40 (SV40) enhancer with a relatively high activity in a broad range of cell lines to viral and cellular enhancers showing very pronounced host cell preferences (for reviews and refs. see 1-3).

The murine retrovirus SL3-3 induces T-cell lymphomas in several tested strains of mice, whereas a very closely related endogenous retrovirus, Akv, is not leukaemogenic (4-6). A recombinant virus, carrying the long terminal repeat (LTR) from SL3-3 and all other parts from Akv, retained the leukaemogenicity of SL3-3 (7). SL3-3 virus grows preferentially in T-lymphocytes, and experiments with recombinant viruses have shown that also this tropism is determined by the LTR (8). The LTRs of SL3-3 and the related Akv virus are identical except for differences in the tandem repeat sequences located about 170 to 370 bp upstream of the start points of transcription and the existence of one extra nucleotide in SL3-3 about 50 bp further upstream. In the LTR of Akv a 99 bp sequence is repeated while the comparable region of SL3-3 consists of two copies of a 72 bp sequence, which has certain similarities with the repeat of Akv, followed by a repetition of the first 34 bp of the repeat (7). The primary determinant of the leukaemogenicity and the T-cell tropism of SL3-3 virus has been proposed to be an enhancer with a preference for T-lymphocytes (9). This idea is supported by two arguments; firstly the DNA sequence differences between the LTR's of SL3-3 and Akv are located far upstream from the transcription start points, in the region of the LTR believed to contain an enhancer (7). Secondly, the U3 region of the LTR of SL3-3 more efficiently enhances the expression of a chloramphenicol acetyl transferase (CAT) gene preferentially in T-lymphocytes than the equivalent region from Akv virus (9). To further investigate the validity of this hypothesis and to delineate DNA sequences important for the activity of the enhancer of SL3-3 virus we have made deletion derivatives of the U3 region. The abilities of the deletion derivatives to stimulate transcription in vivo in T-lymphocyte and other cell lines were measured using a transient assay and quantification of specific RNA by S1 nuclease mapping. The activation of transcription was studied with the heterologous SV 40 promoter including its distal promoter elements (Figure 1) to distinguish SL3-3 enhancer activities from SL3-3 promoter activities.

We report here that the SL3-3 enhancer is composed of many DNA domains whose activities have different importance in different cell lines; each of which is not by itself capable of displaying the full enhancer activity. The repeated DNA sequences were sufficient to retain a large part of the activity of the enhancer in the T-cell lines but not in the other cell lines. We show the existence of multiple enhancer sequence motifs with a high T-lymphocyte specificity in the repeats of SL3-3 virus, the region with many differences compared to the corresponding region of the non-leukaemogenic

Akv virus. Sequence motifs functioning in a broader range of host cells are also identified and located on both sides of the repeated segments of the SL3-3 enhancer.

MATERIALS AND METHODS

Bacterial strains and plasmids

M13 phages were propagated on E. coli strain JM 103 (10). The M13 derivatives M13mp18, M13mp19, M13tg130, M13tg131 and M13mp9A102 (11-13) were used. Plasmids were harvested from E. coli strain 294 (14).

Synthesis of oligonucleotides and other DNA techniques

Oligonucleotides were synthesized by a rapid microscale segmented synthesis method (15) modified for the phosphoamidite chemistry (16). The oligonucleotides were purified on denaturing polyacrylamide gels, phosphorylated, and cloned into linearized M13 vectors by the "shotgun ligation" technique as previously described (13) except that only 0.25 pmol of each phosphorylated oligonucleotide was pooled and lyophilized before ligation. Transformation of E. coli was as described by Hanahan (17). DNA was sequenced with the dideoxy method employing ³⁵S labeling and salt gradient gels (18). Digestions with restriction endonucleases were performed according to the suggestions of the manufacturers, and standard cloning techniques were used throughout.

Cell lines and cell culture

The following cell lines were used ; X 63 Ag8.653 (X 63-1 in text) a nonimmunoglobulin-secreting mouse myeloma (19,20); EL-4, a mouse T-helper cell lymphoma (21,22); CTL, a monoclonal cytotoxic mouse T-cell line (23); and HeLa, a human carcinoma cell line. The lymphocyte cell lines were grown in culture flasks with RPMI 1640 medium supplemented with 5 % fetal calf serum, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 37.5×10^6 units/ml streptomycin, 5.0×10^7 units/ml benzylpenicillin, 1 mM sodium pyruvate and 20 mM β-mercaptoethanol. For CTL, which is dependent on interleukin 2 (IL-2), the medium was supplemented with 40 units/ml of IL-2, which was prepared and assayed as described (24,25). HeLa cells were grown in Dulbecco's modified Eagle's medium (26).

Transfections, RNA preparations and quantitative S1 nuclease mapping

Transfections of the mouse lymphocyte lines were performed as described (27) with slight modifications; 4×10^6 to 6×10^6 cells were centrifugated (1600 rpm, 5 min, TJ-6 Beckman centrifuge), the cell pellet was washed with 10 ml of Tris-buffered saline (TBS; 137 mM NaCl, 25 mM Tris/HCl, 5 mM KCl,

0.7 mM CaCl_2 , 0.5 mM MgCl_2 , 0.6 mM Na_2HPO_4 , pH 7.4), and after a second centrifugation (as above) 2-4 μg of a mixture of recombinant plasmid DNA and reference plasmid DNA (ratio of 1/3) in TBS with 0.5 mg/ml of DEAE-dextran ($\text{Mw } 2 \times 10^6$; Pharmacia) was added to the cells. After 30 min of incubation, the transfection cocktail was removed by centrifugation. For X 63-1 cells an incubation step was also performed with 1 ml of 10% Dimethyl Sulfoxide in TBS for 5 min. The cells were washed twice with TBS and incubated with fresh medium for 40-42 hours. The lymphocytes were harvested by centrifugation (as above). HeLa cells were transfected as described (28).

Preparations of RNA were performed as described (29), with some modifications; frozen cells were suspended in 1 ml of 3 M LiCl, 6 M Urea, 0.1% Sarcosyl, 100 mM β -mercaptoethanol, 50 mM Tris-HCl, 5 mM EDTA, pH 7.5, and sonicated at 0°C . The RNA was precipitated overnight on ice and centrifugated at $14,900 \times g$ (Heraeus microfuge) for 20 min. The pellet was dissolved in 10 mM Tris-HCl, 1 mM EDTA, 0.5% SDS, pH 7.5, deproteinized by two successive phenol extractions and one chloroform extraction and precipitated with ethanol. 10-30 μg of RNA were hybridized with an excess of single stranded 5'-end-labeled DNA probe prepared by extension of a primer from position +60 in the β -globin gene (28), and treated with nuclease S1 (80 units, Amersham) at 25°C for 3-5 hours. The protected DNA sequences were resolved on 6% polyacrylamide/8 M urea gels.

RESULTS

Assay for enhancer activity

We have studied the activity of the SL3-3 enhancer in recombinant plasmids, derived from plasmid pA51 (28), where the coding sequences of the rabbit beta-globin gene are transcribed from the heterologous simian virus 40 (SV 40) promoter (Figure 1A). The effects of the various enhancer mutations were measured by quantitative S1 nuclease analysis of mRNA synthesized during transient expression in different mouse lymphocyte cell lines and, as a comparison, in the human carcinoma cell line HeLa (Figures 1B, 2, 4 and 5). The protected probe DNA sequences, EES and LES, correspond to mRNA initiating at the start sites of the early-early and late-early overlapping promoters, respectively (see 30,31). An internal reference plasmid, pESG 003, with the SV 40 promoter replaced by the beta-globin promoter (Figure 1C), was co-transfected in all experiments. Transcription from this plasmid yielded a signal (Glob in Figures 1C, 2, 4 and 5) whose intensity was used to correct for variations in the efficiencies of different steps of the

FIGURE 1. A. Structure of the plasmids pESG 001, pESG 002 and pESG 100. The enhancer deficient control plasmid pESG 001 is a derivative of pA51 (28) containing the SV40 early promoter region (indicated by a black box) in front of the coding sequence of the rabbit β -globin gene (from position -9 to 1391 (46); double line), in pBR 322 (single line). pESG 001 was constructed from pA51 by deletion of the Nde I fragment between position 1391 of the β -globin DNA and position 2296 of the pBR 322 DNA, followed by a "filling in" of the Apa I site in the β -globin gene (position 1023) using DNA polymerase I (Klenow, large fragment). pESG 002 is a derivative of pESG 001 containing the SV40 enhancer with one copy of the 72 base pair repeat. In pESG 100 the DNA region upstream of the "TATA" box of the LTR of SL3-3, from position 1 to 446 (7), with Kpn I and Bam HI sites engineered at the respective ends, was cloned in front of the SV40 early promoter (see also Figure 3). B and C. Determination of transcriptional activity of enhancer DNA on the SV40 early promoter. The amount of RNA synthesized from the SV40 early promoter in the pESG-type recombinants after transient expression in HeLa cells or mouse lymphocyte cell lines, was determined by quantitative S1 nuclease mapping. B. Part of the pESG series of recombinant plasmids with the SV40 promoter region (Bam HI to Hind III; single line) cloned in front of the rabbit β -globin coding sequences (double line). EES and LES represent single-stranded DNA probe fragments of 130 to 137 and 164 to 178 nucleotides in length, respectively, protected against S1 nuclease digestion by RNA initiating at the SV40 start sites EES and LES, respectively. C. Part of the recombinant pESG 003 which has the SV40 early promoter replaced by the β -globin promoter and was used as a reference plasmid in the transient expression assay. The Pst I-Bam HI fragment containing most of the rabbit β -globin promoter of plasmid p β (244⁺) β (47), and a Bam HI-Pst I linker sequence, 5'-GATCCAGACCTCACCCTGCA-3', completing the β -globin promoter, were used to replace the Bam HI fragment containing the SV40 promoter. The TATA box and the start site of the β -globin gene (double line) are indicated. Glob represents probe fragments protected against S1 nuclease digestion by RNA initiating at the globin cap site (60 nucleotides in length).

transcription assay. The effect of enhancer mutations on transcription initiated at the LES was not systematically studied here, but appeared to follow those of transcription initiated at the EES as expected from previous studies (28,32,33).

Cell type preference in stimulation of transcription by the SL3-3 enhancer

As an initial step in our analysis of the SL3-3 enhancer the whole DNA region upstream of the "TATA" box of the LTR of SL3-3 virus (nucleotide positions 1 to 446; Figure 1A; numbering system of Lenz et al. (7)), believed to contain the enhancer (9), was constructed from oligonucleotides (Materials and Methods). In outline, a set of DNA segments, representing the different parts of this DNA region, and bordered by restriction enzyme cleavage sites (Figures 1A and 3), were assembled from overlapping oligonucleotides and cloned into derivatives of phage M13 by the "shot-gun ligation" technique (13). The DNA segments were subsequently, by several subcloning steps, assembled into the complete DNA region of SL3-3 and placed in front of the SV 40 promoters and the rabbit beta-globin structural gene (Figure 1A). The ability of the constructed plasmid, pESG 100, to stimulate transcription compared to the corresponding plasmid without enhancer, pESG 001, was analysed in three mouse lymphocyte cell lines: CTL, a monoclonal cytotoxic T-cell line; EL-4, a T-helper-cell lymphoma, and X 63-1, a non-immunoglobulin secreting cell of the B lineage, and, as a control for species and/or cell type specificity, also in HeLa, a human cell line of non-haematopoietic origin. The 446 bp region from SL3-3 virus was found to efficiently stimulate transcription from the SV 40 early promoters in all four cell lines studied (cf. lanes 1, 4, 7 and 10 with lanes 3, 6, 9 and 12 of Figure 2) showing, with an assay at the RNA level, that this DNA region from SL3-3 contains an element enhancing transcription from a heterologous promoter. The enhancer activity obtained was much higher than that with a homologous plasmid containing the SV 40 enhancer (pESG 002, a derivative with one copy of the 72 bp segment; Figure 1A) in both T-cell lines used while the SV 40 enhancer was the more active in the non-T-cell lines (cf. lanes 1, 4, 7 and 10 with 2, 5, 8 and 11 of Figure 2). The results of many experiments such as those shown in Figure 2 are summarized in the upper part of Table 1. The enhancer activity of the region from SL3-3 was found to be seven fold higher than that of the SV 40 enhancer in CTL and 1.6 fold higher in EL-4. In contrast, the SV 40 enhancer was 1.5 fold more efficient than the SL3-3 enhancer in the B-cell line and 3.7 fold more efficient in HeLa. Thus, SL3-3 contains an enhancer which, relative to the broad host range enhancer of SV

FIGURE 2. Quantitative S1 nuclease analysis of RNA synthesized by: 1, 4, 7 and 10 - pESG 100, containing the SL3-3 enhancer. 2, 5, 8 and 11 - pESG 002, containing the SV40 enhancer. 3, 6, 9 and 12 - pESG 001, lacking enhancer, transfected into HeLa, X 63-1, EL-4 and CTL cells, respectively. EES corresponds to RNA transcribed from the SV40 early promoter and Glob to RNA transcribed from the co-transfected reference recombinant pESG 003 (see text and Figure 1B and C).

40, shows a T-cell preference. These results obtained with an assay directly at the RNA level confirm the results obtained by Celander and Haseltine with a CAT assay (9).

Enhancer domains with cell type differences in their effects

To delineate DNA sequences required for the activity of the SL3-3 enhancer, we constructed a series of deletion mutants having restriction fragments of the enhancer (Figure 3) replaced by shorter DNA segments (Materials and Methods). Deletion of the DNA sequences upstream of the repeated DNA segments (pESG 106, pESG 107 and pESG 108, Figure 3) had a relatively small effect on the level of transcription in all the four cell lines (Figure 4 and Table 1). With the largest deletion, pESG 106, lacking the whole DNA segment upstream of the repeats, the transcription decreased to between 41 and 74 per cent of pESG 100 for the different cell lines, with the smal-

FIGURE 3. Physical map of various deletion mutants of the SL3-3 enhancer. Dots indicate deleted sequences. The DNA segments used in the construction of the pESG variants were constructed by the "shot-gun ligation" technique (13). 51 different oligonucleotides, designed with at least 8 bases overlap, were ligated to sets of DNA segments bordered by the naturally occurring Pst I, Apa I, Ava II (positions 155, 227 and 299; not indicated) and Sau3AI restriction enzyme cleavage sites, and the artificial Kpn I and Bam HI restriction enzyme sites. DNA segments having Bam HI sites in the downstream end were cloned into M13mp9A102 (13), while other DNA segments were cloned into M13mp18, M13mp19, M13tg130 or M13tg131. The DNA segments were assembled into the shown DNA regions in several subcloning steps using standard cloning techniques. The DNA regions were subsequently placed in front of the SV40 promoter of pESG 001. The sequences of all recombinants were confirmed by DNA sequencing.

least effect found for the cytotoxic T-cell line, CTL. The construct with the deletion extending to position 237, pESG 115, and therefore retaining only one copy of each of the 34 bp and 38 bp DNA segments constituting the 72 bp repeat in the wild type, did not result in a significant further decrease in the level of transcription in the lymphoid cells but in HeLa cells only 22 per cent of the activity remained (Table 1). Deletion of all DNA sequences upstream of the Apa I site at position 271 (plasmid pESG 113), including the

Table 1. Effect in different cell lines of deletions within the enhancer of SL3-3 virus

Recombinant	Relative transcription (%)			
	CTL	EL-4	X 63-1	HeLa
pESG 100	100	100	100	100
pESG 001	2	3	3	0.5
pESG 002	15	61	150	370
pESG 106	74	41	55	56
pESG 107	104	57	47	82
pESG 108	107	80	64	51
pESG 110	56	78	35	8
pESG 111	73	47	13	4
pESG 112	91	61	6	4
pESG 113	26	9	37	24
pESG 115	76	40	46	22
pESG 116	42	70	72	ND
pESG 124	42	43	50	85
pESG 126	68	91	81	72
pESG 127	106	87	46	ND
pESG 134	103	146	80	108
pESG 145	9	ND	ND	22
pESG 53	36	27	2	4

The effect of deletions within the enhancer region of SL3-3 virus on transcription initiating from the EES of the SV 40 early promoter in four different cell lines (Materials and Methods). All results are expressed relative to pESG 100 in that cell line, taken as 100 %. The assay of transcription, the recombinants and their construction are described in Figures 1, 2 and 3. The values which represent the average ($\pm 20\%$) of several independent transfection experiments with different plasmid DNA preparations were obtained by densitometric scanning of autoradiograms similar to those shown in Figures 2, 4 and 5. ND, not determined.

38 bp DNA segment repeated twice in the wild type and only leaving one copy of the three times repeated 34 bp segment, decreased transcription about 3 to 4 fold further in both T-lymphocyte cell lines while transcription from this plasmid was not affected more than from pESG 115 in the human carcinoma line, HeLa, and only slightly more in the B-cell line (Table 1). A construct with a deletion extending to position 320, pESG 145, and therefore lacking

FIGURE 4. Effect of deletions in the SL3-3 enhancer on RNA synthesis after transfection into four different cell lines: HeLa, X 63-1, EL-4 and CTL, as measured with quantitative S1 nuclease mapping. 1,2,3,4,5 6 and 7 at the top of each lane represents the recombinant plasmids; pESG 106, pESG 107, pESG 108, pESG 100, pESG 110, pESG 111 and pESG 112, respectively. EES corresponds to RNA transcribed from the start site of the SV40 early promoter and Glob to RNA transcribed from the co-transfected reference recombinant pESG 003 (see text and Figure 1B and C).

all 38 and 34 bp repeats; resulted in a 3 fold larger decrease in transcription in CTL than the deletion to position 271, but about the same level of transcription as pESG 113 and pESG 115 in HeLa cells.

The severe effect on transcription obtained in the T-lymphocyte cell lines by deleting the 38 bp sequence could be partially reversed by including the DNA sequences upstream of the repeats in the construct (cf. pESG 113, pESG 115, pESG 124 and pESG 126 in Table 1). Also, the drastic decrease in transcription obtained in HeLa by deletions from the upstream side including most of the repeats of the 34 and 38 bp DNA segments was clearly reduced by readdition of the DNA upstream of position 130 (cf. pESG 113 and pESG 115 with pESG 124 and pESG 126 in Table 1). Interestingly, with the upstream region present, the deletion of the remaining 38 bp segment of pESG 126 decreases the transcription up to two fold in the lymphoid cell lines while transcription was not at all affected in HeLa. The 38 bp DNA segment could be inverted with at least a large part of its function kept (cf. pESG 115, pESG 116, pESG 126 and pESG 127 in Table 1). It is notable that deletion of

only one copy of each of the 34 and 38 bp repeats (pESG 134) did not significantly decrease transcription. In EL-4 this construct even showed increased enhancer activity. These results are probably obtained because of sequence redundancy within the repeats and because the deletion brings the upstream enhancer domains closer to the rest of the enhancer and the promoter.

Deletion of the DNA sequences downstream of the repeats (pESG 110, pESG 111 and pESG 112, Figure 3) had little effect on the level of transcription in the T-lymphocyte cell lines, CTL and EL-4 (Figure 4 and Table 1). In contrast, the same deletions drastically decreased the level of transcription in the two other cell lines, X 63-1 and HeLa. The decrease was more pronounced for both cell lines with the two largest deletions than with the smallest deletion; In X 63-1, the deletions ending at positions 379, 336 and 311 decreased transcription about 3, 8 and 17 fold, respectively. The same deletions decreased transcription more in the HeLa cells, about 12, 25 and 25 fold, respectively. Thus, several DNA elements (the difference in transcription with pESG 110, pESG 111 and pESG 112) downstream of the DNA repeats are clearly much more important in the non-T-cell lines than in the T-cell lines. Note in this context that in X 63-1 the deletion of the DNA downstream of position 310 decreases transcription close to the level of the plasmid lacking enhancer and clearly much more than the deletion of the 38 bp sequence and all DNA upstream of it (cf. pESG 001, pESG 112 and pESG 113 in Table 1).

To study if the repeated region by itself could enhance transcription in the T-cell lines deletions of the sequences upstream and downstream of the repeated region, which had relatively low effects on transcription, were combined. Such a construct, pESG 53 (Figure 3), resulted in a level of transcription only about three fold lower than that from the wild type in the T-cell lines (cf. pESG 100 and pESG 53 in Table 1). This shows that the repeated sequences of SL3-3 clearly can stimulate transcription by themselves in these cell lines although the sequences not are as efficient as when combined with either the nearby upstream or downstream sequences of the enhancer (cf. pESG 53, pESG 106 and pESG 112 in Table 1). The T-cell specificity of the activity of the repeated region without the normal surrounding sequences is very high. The transcription in EL-4 is only two fold lower from pESG 53 than from the SV 40 enhancer (pESG 002), and in CTL even 2.5 times higher from pESG 53. However, the transcription in X 63-1 and HeLa is 75 to 92 fold lower from pESG 53 than from the broad range SV40 enhancer.

FIGURE 5. Quantitative S1 nuclease analysis of RNA from HeLa cells transfected by pESG 100, pESG 106 and pESG 145 (Figure 3), with (+) or without (-) the addition of 1 μ M dexamethasone (final concentration) 24 hours after addition of the DNA. EES corresponds to RNA transcribed from the SV40 early promoter and Glob to RNA transcribed from the co-transfected reference recombinant (see text and Figure 1B and C). Note the much stronger globin control band of pESG 145, autoradiography times were chosen for convenient comparison of the dexamethasone responses.

The transcription from pESG 53 in X 63-1 was at the level of the construct lacking enhancer, pESG 001.

A glucocorticoid-responsive element in the repeated sequences

In a recent report (34), the enhancement of expression of a reporter gene (chloramphenicol acetyl transferase (CAT)) from the LTR of SL3-3 virus was shown to be increased in HeLa cells (but not in the T-lymphocyte line studied) by the synthetic glucocorticoid dexamethasone. A sequence within the 38 bp repeat, 5'-AGAACAGATGGTCCCCA-3', with homology to the consensus sequence for glucocorticoid receptor binding sites (35,36), was proposed to be the glucocorticoid-responsive element (GRE). We have reported above that deletion of the only remaining copy of the 34 and 38 bp segments did not decrease the activity of the non-induced enhancer in HeLa cells (cf. pESG 115, pESG 113 and pESG 145). We find that dexamethasone increases the activity of the SL3-3 enhancer in HeLa and also, although to a lower extent, in several lymphocyte cell lines (Figure 5 and data not shown). In order to test if the GRE was indeed the homology seen in the 38 bp repeat, or alternatively a homology located upstream or downstream of the repeats in the enhancer (eg. positions 98 to 114, 88 to 72 or 319 to 335), we compared the effect of adding dexamethasone on the transient expression from pESG 100,

pESG 106 and pESG 145 (Figure 5). A 3 to 4 fold stimulation by the glucocorticoid was reproducibly seen for both pESG 100 and pESG 106 while no effect of dexamethasone was seen for pESG 145. This shows that the repeated sequences, which without induction did not mediate significant enhancer activity in HeLa cells, contain a glucocorticoid-responsive element.

DISCUSSION

In the present study we clearly demonstrate that different DNA sequences located upstream of the "TATA" box in the LTR of the retrovirus SL3-3 could enhance transcription from a heterologous promoter complete with all its distal promoter elements, a property generally attributed to enhancer elements (1-3). Another property of enhancers is the ability to retain a substantial part of their activity when located at a distance from the promoter. The SL3-3 sequences from position 41 to 378 have previously been reported to stimulate expression from a chloramphenicol acetyl transferase (CAT) gene under the control of the SV40 promoter when situated far away (9). The most essential sequences for enhancement of transcription in T-lymphocytes were found to be located about 170 to 350 bp upstream of the mRNA start site, and in non-T-cells a substantial part of the enhancer activity was located far from the promoter. Nevertheless, no apparent increase in transcription was seen in any of the different constructs when the distance to the promoter was decreased (Figures 3 and 4 and Table 1). This argues that the ability of these DNA sequences to enhance transcription is relatively insensitive to their distance from the promoter. In this study we also compared the enhancer activity of the SL3-3 enhancer and that of SV 40, which has been shown to be active in a broad range of cells. The SL3-3 enhancer was found to be stronger than the SV 40 enhancer in the T-cell lines while the opposite was the case in the other cell lines used, showing that the SL3-3 enhancer has a strong T-cell preference relative to this enhancer (Figure 2 and top of Table 1). However, the different absolute levels of enhancements obtained with the SL3-3 enhancer could be affected by differences in the ability to enhance the particular heterologous promoter used, since it has been reported that an enhancer can stimulate a particular promoter much more than other promoters (37).

Our results show that the SL3-3 enhancer is composed of several DNA domains none of which by itself displays the full enhancer activity. These domains appear to be of different importance in the different host cell lines. The low contribution of the DNA segments downstream of the repeats to the enhancer activity in T-cells (Figure 4 and Table 1) could mean that

these DNA segments have very little activity in these cell lines, or alternatively, that the enhancer activity of the 34 bp and 38 bp repeat alone is so high that the downstream DNA segments can not improve the transcriptional capacity much further. The almost 5 fold higher transcriptional activity in CTL of a construct containing only the downstream DNA segments when compared to the corresponding plasmid without enhancer strongly supports the second alternative (cf. pESG 145 and pESG 001 in Table 1). The step-wise decrease in the enhancer activity in the non-T-cell lines with the deletions from the downstream side extending to positions 379, 335 and 310, argues for the existence of several enhancer domains downstream of the repeats. The localisation of enhancer domains in this segment is supported by the result that most of the enhancer activity in HeLa cells of the construct with a deletion of the SL3-3 DNA upstream of position 320, pESG 145, was lost when the deletion was extended to position 362 (data not shown). The DNA segments found to be very important for the SL3-3 enhancer activity in the non-T-cells reveal high homology to the TC-motifs of the simian virus 40 enhancer (28). A 52 kD nuclear enhancer binding protein which was denoted AP-2 and recognizes the TC-motifs of SV 40 has been purified (38). Together with other identified binding sites for AP-2 a consensus binding sequence, 5'-CCCCAGGC-3', has been proposed (38). Several sequences downstream of the repeats in the SL3-3 enhancer (positions 360-367, 375-368, and 381-388) and the sequence 5'-CCCCAGAC-3', overlapping the border between the 34 bp and 38 bp repeats, all show six to seven nucleotides homology to this consensus sequence. Deletion of most of the 34 and 38 bp repeats leaving one of each decreased transcription differentially in HeLa cells (cf. pESG 115 and pESG 106 in Table 1), and deletion of the remaining repeats did not decrease transcription further (cf. pESG 115, pESG 113 and pESG 145 in Table 1) This argues that the borders between the 34 bp and 38 bp repeats deleted in pESG 115 but not in pESG 106 contains a sequence contributing to the activity of the enhancer. Divergences from the consensus binding sequence of AP-2 have, in different known binding sites been found at all positions where the four noted sequence motifs of SL3-3 differ from the consensus. It is therefore probable that these motifs of the SL3-3 enhancer indeed bind the enhancer protein AP-2 or a related protein.

Although deletion of the SL3-3 DNA upstream of the DNA repeats had only a small effect on the activity of the otherwise complete enhancer, this DNA segment had a high ability to compensate, both in the T-lymphocytes and the non-T-cells studied, for deletions of 34 bp and 38 bp repeat sequences

(Table 1). An apparent feature of the DNA segments upstream of the 34 and 38 bp repeats is a series of poly(dA-dT) homopolymer sequences, at positions 50-53, 66-70, 92-96 (continued with mostly dA-dT basepairs to position 105) and 123-127. The upper strand of the 41 bp closest to the repeats contains about 60 per cent A residues. Poly(dA-dT) homopolymer sequences have been shown to mediate a structure of the DNA distinct from that of typical B DNA (39 and refs. therein). Such homopolymer sequences are constitutive elements found to be important for the expression of several eucaryotic genes and have been proposed to act by disfavours nucleosome formation (40). Such a mechanism of action could fit with the observation that larger effects of the upstream DNA segments were observed when important elements of the enhancer were deleted (cf. pESG 124 with pESG 113 and pESG 106 in Table 1). It is possible that it then becomes more important to avoid formation of nucleosomes so that factors can interact with the remaining binding sites compared to when additional enhancer regions are present and other binding proteins can cooperate in excluding formation of nucleosomes. However, the possibility that specific enhancer binding proteins interact with sequences upstream of the repeats and mediate all or a part of the activity of this region of the enhancer can not at present be excluded.

The repeated DNA sequences of SL3-3 by themselves only gave one to two per cent of the transcriptional activity of the SV40 enhancer in the non-T-cells, while the same sequences showed half of the activity of the SV40 enhancer in the T-helper cell line, EL-4, and 2.5 fold times higher activity than this enhancer in the cytotoxic T-cell line (cf. pESG 53 and pESG 002 in Table 1). The results show the existence of enhancer sequences with a high preferential activity in T-lymphocytes, located in the 34 bp and 38 bp repeats of SL3-3 virus. Both the 34 bp and 38 bp repeats were found to mediate enhancer activity in the mouse lymphocyte cell lines but not in HeLa (cf. pESG 115, pESG 113 and pESG 145 in Table 1). In a separate report we have shown data that addresses the question of which DNA sequences are involved in the differential enhancer activity of the repeats (41). We have identified a group of proteins, denoted SL3-3 enhancer factor 1 proteins (SEF1) which specifically bind to a DNA sequence, including but not limited to 5'-CTGTGGTTAA-3', within the 38 bp repeat (positions 183-192 and 255-264). A mutation in this binding site, which abolished binding of SEF1 in vitro, decreased transcription in vivo two fold in the T-cell lines while no decrease was obtained in X 63-1. The SEF1 proteins were found to be much more abundant in T-cells than in other cell types studied. These results

strongly argue that the activity of the 38 bp repeat seen in the T-cell lines is mediated by the binding of SEF1 proteins. The enhancer of Akv, a very closely related virus which does not show T-cell tropism or cause T-cell lymphomas, is different from that of SL3-3 in the SEF1-binding site (41). A DNA segment with the SL3-3 sequence binds SEF1 much better than a segment with the Akv sequence. Thus, the differences in T-cell preference, both in the viruses and in their enhancers, correlates with differences in the efficiencies of the DNA sequences of SL3-3 and Akv studied to bind to a transcription factor, SEF1, that is preferentially made in T cells (41). However, the total effect of the repeats on transcription in vivo is much more than two fold in the lymphocyte cell lines. In addition, deletion of the last 38 bp repeat also decreased transcription in the myeloma cell line, X 63-1, although to a lesser extent than in the T-cell lines (Table 1). This argues for the existence of additional enhancer motifs, both with and without T-cell preferences, in the repeated region.

Nuclear factor I (NFI) is a protein found to enhance initiation of replication of adenovirus DNA (42) and the transcription of many genes (43 and refs. therein). NFI binding sites of a number of genes have been compared (44-46). The nucleotide sequence 5'-CCGGCCCAGGGCCAA-3' in the 34 bp repeat of the SL3-3 enhancer (position 133-146, 205-218 and 277-290) shows a high degree of homology with these binding sequences of NFI. In a separate report we show that binding of NFI to this sequence contributes to the activity of the SL3-3 enhancer in vivo to different degrees in different lymphoid cell lines but not in HeLa cells (Nilsson et al., manuscript in preparation).

In the present report we confirm the observation of Celander and Haseltine (34) that the synthetic glucocorticoid dexamethasone stimulates the enhancer of SL3-3. Interestingly, we could localize the glucocorticoid-responsive element (GRE) to the repeated sequences, which without induction did not mediate any significant enhancer activity in HeLa cells. Thus, the domains important for the basal level enhancer activity in HeLa cells are located separately from the GRE.

A major conclusion from the present report is that sequences mediating preferential activity of the SL3-3 enhancer in T-cells are located within the repeated sequences of the U3 region of the LTR. These repeats contain all the differences, except for one extra A residue in the poly A-stretch at positions 66-70, between the LTR's of SL3-3 and the very closely related virus Akv, which does not show T-cell tropism or cause T-cell lymphomas (4-

8). The results of the present report support the notion that enhancer sequence motifs within the repeats, with T-cell preferences in their activity, are primary determinants of the T-cell tropism and the leukaemogenicity of the SL3-3 virus.

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