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Interaction of Viral and Cellular Factors with the HTLV-III * LTR Target Sequences In Vitro

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A. Summary

The location of *cis*-acting regulatory sequences within the long terminal repeat (LTR) of the human T-cell lymphotropic virus type III was determined by eukaryotic cell transfection and chloramphenicol acetyltransferase (CAT) assay or in vitro cell-free transcription. A 160 base pair (bp) region of the LTR at position −104 to 56 is required for *trans*-activation (cap site 1). A 24 bp enhancer element (EHE) capable of increasing the rate of transcription, irrespective of orientation, is located between nucleotides −105 to −80. It contains two 10 bp repeats. Three Sp1 binding sites (Sp1 III-I) are located between −78 and −45. A deletion of Sp1 III allowed for limited TATIII response while the presence of a functional enhancer restored the activity in HTLV-III infected cells. Complete loss of transcriptional activity and CAT gene expression could be attributed to the absence of EHE and Sp1 III-I at position −48. However, reinsertion of the enhancer restored accurate initiation but at a decreased level suggesting that the presence of a Sp1 binding site is not a prerequisite for the accurate initiation of transcription but is required for transcriptional activation independent of a promoter. The presence of a negative regulatory element (NRE) has been demonstrated by removal of the 5′ part of U3 to position −117.

Nucleotide sequences around the cap site and poly (A) site contain a *trans*-activator response element (TRE) and could be arranged into a unique secondary structure. A deletion of four nucleotides TCTGAGCCTGGG*AGCTC* causes a loss of three dimer linkage sequence binding. The CAT gene enzyme expression is completely abolished but transcriptional activity remains at reduced level.

B. Introduction

Human T-lymphotropic virus type III (HTLV-III, LAV-1, HIV), the virus causing the acquired immunodeficiency syndrome (AIDS), can infect OKT 4+ human T-cell lines in culture [13]. A high level of virus expression is observed after 1 week. This high gene expression results, at least in part, from transcriptional [7] and post-transcriptional activation [2, 9]. In both mechanisms the virus encoded *trans*-activator protein TATIII is involved. The target site of this protein (*trans*-acting responsive element, TAR) is part of the LTR downstream of the RNA initiation site [10]. Other regulatory elements within the LTR have been detected: a negative regulatory element (NRE), an EHE [10], and three binding sites for the Sp1 protein[4].

In this study various deletion mutants of the LTR have been tested to locate *cis*-acting regulatory regions responsive to the virus associated *trans*-acting regulatory factors at transcriptional and post-transcriptional levels.

* HTLV-III/LAV = HIV

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C. Material and Methods

I. Cell Lines

H9 and HTLV-III infected human H9 T-lymphocytes (H9/III) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Hela cells, S3, were grown in MEM with 10% FBS.

II. Plasmid Constructions

Plasmids pSVOCAT, pSV2CAT, and pRSVCAT have been previously described [11]. pC15CAT (Fig. 1) was constructed by blunt ending the *Pst*I cDNA insert of C15 [1], ligation of *Hin*dIIIlinkers, and and subsequent ligation of the resulting fragment into the *Hin*dIII site of pSVOCAT. The 5′ deletion mutants were derived from pC15CAT, which contains a portion of 3′ orf, U3 and the majority of the R region, by cleavage at the *Kpn*I site, digestion with *Bal*31 exonuclease, blunt-ending and the addition of a unique *Xba*I site. These 5′ mutants include pCD12CAT (no deletion within the LTR, ca. 50 bp, *Knp*I site), pCD7CAT (deletion to −278), pCD16CAT (−176), pCD23CAT (−117), pCD52CAT (−65), and pCD54CAT (−48). Nucleotide 1 was assigned as determined by nuclease S1 mapping [12]. Synthetic oligonucleotides of HTLV-III sequences (EHE) from −105 to

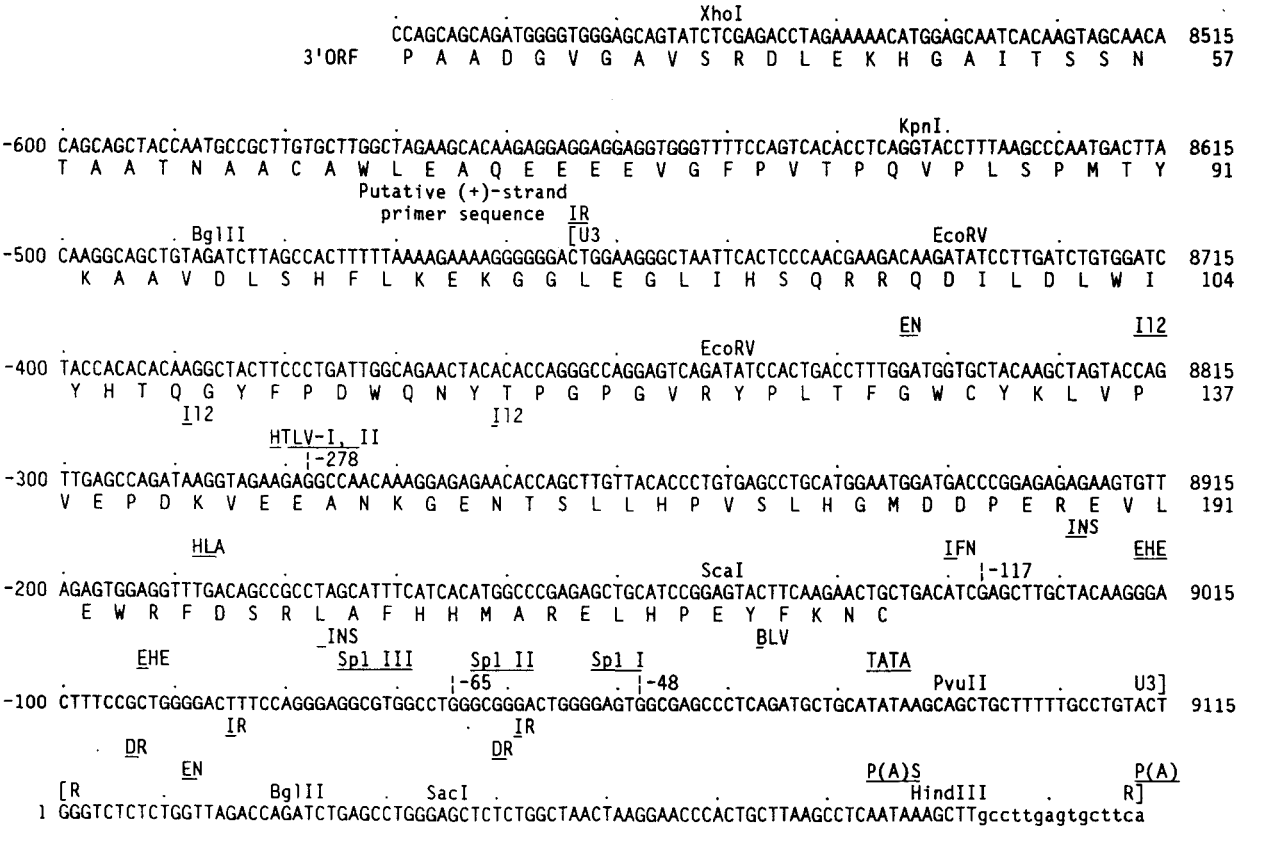

Fig. 1. Sequence of pC15CAT. Numbers on the right of each line are according to Ratner et al. [8]. The 5′ deletion clones schematized in Fig. 2a and b are indicated by vertical lines followed by the distance of each from the transcriptional start site at 1 (position 9116). Shown are the portion of the coding region for 3′ orf which terminates at position −125 (8991). The LTR sequences begin at −454 bp (8662) from the CAP site. The promoter signal (TATA) is at −27 and the polyadenylation signal [P(A)S] at position 74. Bases written in lower case letters from 84 to the polyadenylation site [P(A)] at 98 were deleted from the C15 insert during the construction of C15CAT and replaced by the bacterial chloramphenicol transferase gene. Sequences homologous to other genes as previously reported and EN are underlined. *IL-2*, interleukin 2; *HTLV-I, -II*, human T-lymphotropic viruses type I and II; *IF*, gamma interferon; *INS*, insulin gene; *EHE*, enhancer; *Sp1 III-I*, Sp1 binding sites (−77 to −46); *EN*, core enhancer consensus sequences; and *DR* or *IR*, direct or inverted repeats

−80 were placed in front of pCD52CAT (−65) and pCD54CAT (−48) in sense orientation which results in pCD52ECAT and pCD54ECAT (*GGGACTTTCC*GCTG *GGGACTTTCC*ATCTAGA −65 or −48), or antisense orientation which results in pCD52EWCAT and pCD54EWCAT (*GGAAAGTCCC*CAGC*GGAAAGTCCC*T-CTAGA −65 or −48). pVHHCAT (5′–3′) and pVHHWCAT (3′–5′) were constructed by cloning the 3′ *Hin*dIII fragment of pHXB2gpt [3] into pSVOCAT. DNA templates for in vitro transcription were prepared by cleavage with *Nco*I. For the deletion mutants within the TRE, pCD23CAT was digested with *Sac*I (pCD23ΔSCAT), *Bgl*II and *Sac*I (pCD23ΔBSCAT), *Bgl*II and *Hin*dIII (pCD23ΔBHCAT), or *Sac*I and *Hin*dIII (pCD23ΔSHCAT) and self ligated. pCD23ΔSHaCAT is a replacement of the *Sac*I and *Hin*dIII fragment (38 to 80) by a oligonucleotide sequence from 39 to 56. The extent of each deletion was confirmed by DNA sequencing analysis [6] and revealed that the resulting mutants pCD23ΔSCAT and pCD23ΔSHaCT lacked, after ligation, the *Hin*dIII fragment containing tailing sequences.

III. Eukaryotic Cell Transfections and CAT Assays

In general, 10 μg of plasmid DNA was transfected into approximately 10^7 lymphocytes using the DEAE-dextran method. Cell extracts were prepared by three freeze and thaw cycles 48 h post-transfection. CAT assays were performed as described previously [11].

IV. In Vitro Transcription

Nuclear extracts were prepared from H9 HTLV-III infected cells (H9/IIIE) [7] and whole cell extracts from Hela cells (Hela CE) [5]. Standard in vitro transcription mixtures (25 μl) contained 12 m*M* HEPES (pH 7.9), 60 m*M* KCL, 7 m*M* MgCl, 0.2 m*M* EDTA, 1.3 m*M* DTT, 10% glycerol, 50 μM each of ATP, CTP, and UTP, 5 μ*M* GTP plus 10 μCi of [$^{-32}$P] GTP, 4 m*M* creatine phosphate, 0.4 μg of poly [d(I-C)]: poly [d(I-C)] as a carrier, 10 μl of Hela whole cell extract (Hela CE), and 0.4 μg (16 μg/ml) of template DNA. Extraction of RNA products, denaturation with glyoxal, and agarose (1.8%) gel electrophoresis followed. To quantitate the in vitro transcripts, gel slices were cut and the radioactivity was counted.

D. Results

To identify transcriptional regulatory elements, recombinant plasmids containing the bacterial chloramphenicol acetyltransferase (CAT) gene with various deletions from the 5′ end of the HTLV-III LTR, detailed in Fig. 1, were investigated in an in vitro cell-free system or transfected into H9 or infected H9 cells for CAT enzyme level determination.

In a second series of deletions, we examined nucleotide sequences surrounding a possible hairpin structure in the terminal redundancy (R). Table 1 summarizes the results of these experiments.

I. Identification of Negative Regulatory Regions

The activity of the individual 5′ LTR deletion mutants was tested by co-transfection experiments with pHXB2gpt, transfection into H9 or H9/III, or by in vitro transcription. A small deletion of ca. 50 nucleotides (pCD12CAT) into the 3′orf sequences of pC15CAT significantly increased the level of CAT gene expression in co-transfection (twofold) and transfection (fourfold) experiments. Additional deletions from −278 (pCD7CAT) to −117 (pCD23CAT) showed no further increase. However, we also examined a plasmid, pVHHCAT which contains the 3′ end of envelope, 3′orf, U3, and R sequences derived from an infectious provirus. In contrast to pC15CAT, the level of CAT gene expression was increased and values are comparable to pCD12CAT (Table 1, Experiment 2). To determine if the level of CAT enzyme activity reflects their in vitro transcriptional activities, we tested pCD12CAT, pCD7CAT, pCD16CAT, and pCD23CAT in an in vitro cell-free system with or without preincubation of nuclear ex-

Tabelle 1. CAT activity and *in vitro* transcription activity of LTR recombinants and controls

Plasmid	Description	Co-transfection with pHXB2gpt(a)	Absolute CAT activity				*In vitro* transcription activity (c)	
			Exp 1 (b)		Exp 2 (b)			
		H9	H9	H9III	H9	H9III	Hela CE	
	(30 min)	(16 h)	(30 min)	(15 h)	(20 min)	H9	H9	H9/IIIE
pVHHCAT	pHXB2gpt(5'-3')	NT	NT	NT	2.4±0.34	92.7± 1.3	NT	NT
pC15CAT	C15	24.1 (0.0)	0.3	5.1±0.5	0.1±0.0	20.8	NT	NT
pCD12CAT	Del KpnI	44.9	2.0	39.4±5.9	0.7±1	88.8±10.9	2	8
pCD7CAT	-278	46.4	2.6±0.3	58.0±9.8	NT	NT	2	9
pCD16CAT	-176	58.4	2.1±0.3	60.5±12.3	NT	NT	3	9
pCD23CAT	-117	21.4	1.1±0.1	55.9±3.2	0.4	97.5	4	10
pCD52CAT	-65	2.1	0.0	1.6±0.5	0.1±0.0	32.1±0.0	1	2
pCD52ECAT	EHE(5'-3')-65	NT	NT	NT	0.4±1.0	99.0± 0.0	NT	7
pCD52EWCAT	EHE(3'-5')-65	NT	NT	NT	0.9±0.3	99.2± 0.1	NT	NT
pCD54CAT	-48	0.0	0.0	0.0	0.0±0.0	0.0± 0.0	0	1
pCD54ECAT	EHE(5'-3')-48	NT	NT	NT	0.2±0.0	26.7± 0.0	NT	4
pCD54EWCAT	EHE(3'-5')-48	NT	NT	NT	0.2±0.0	30.8± 1.0	NT	NT
pCD23ΔSHaCAT	-117 to 56	NT	1.0	40	NT	NT	1	7
pCD23ΔSHCAT	-117 SacI-HindIII	NT	1.0	0.0	16.7	1.2± 0.3	NT	NT
pCD23ΔBHCAT	-117 BglII-HindIII	NT	NT	NT	0.1	0.0	0	3
pCD23ΔSCAT	-117 SacI	NT	NT	NT	0.2	0.0	1	4
pCD23ΔBSCAT	BglII-SacI	NT	1.0	0.0	0.2	0.1	0	2
pVHHWCAT	pHXB2gpt(3'-5')	NT	NT	NT	0.1±0.0	0.0	NT	NT
pSVOCAT	CAT	NT	NT	NT	0.0±0.0	0.0± 0.0	NT	NT
pSV2CAT	SV 40	NT	NT	NT	6.7±1.5	2.6± 0.0	NT	NT
pRSVCAT	RSV	(2.9)	61.0	0.5±0.1	48.4±3.4	18.8± 4.8	NT	NT

[a] Co-transfection using HTLV-III LTR CAT plasmids and pHXB2gpt in H9 cells. Absolute activies are average valuese for two independent experiments 48 h post-transfection. pC15CAT and pRSVCAT values in parentesis are transfections without pHXB2gpt.

[b] Plasmids were transfected into the indicated cells and CAT assays were performed on extracts after 48 h. The values show percent conversion per time of chloramphenicol to acetylated metabolites. Transfections were done in triplicate.

[c] Ratio of CAT enzyme activities of H9 (16 or 15 h) and H9/III (30 or 20 min).

[d] Run-off RNAs were obtained from in vitro transcription of indicated plasmids after digestion with *Nco*I. Values indicate relative radioactivity between 0 and 10.

NT, not tested; Exp, Experiment; Del, deletion.

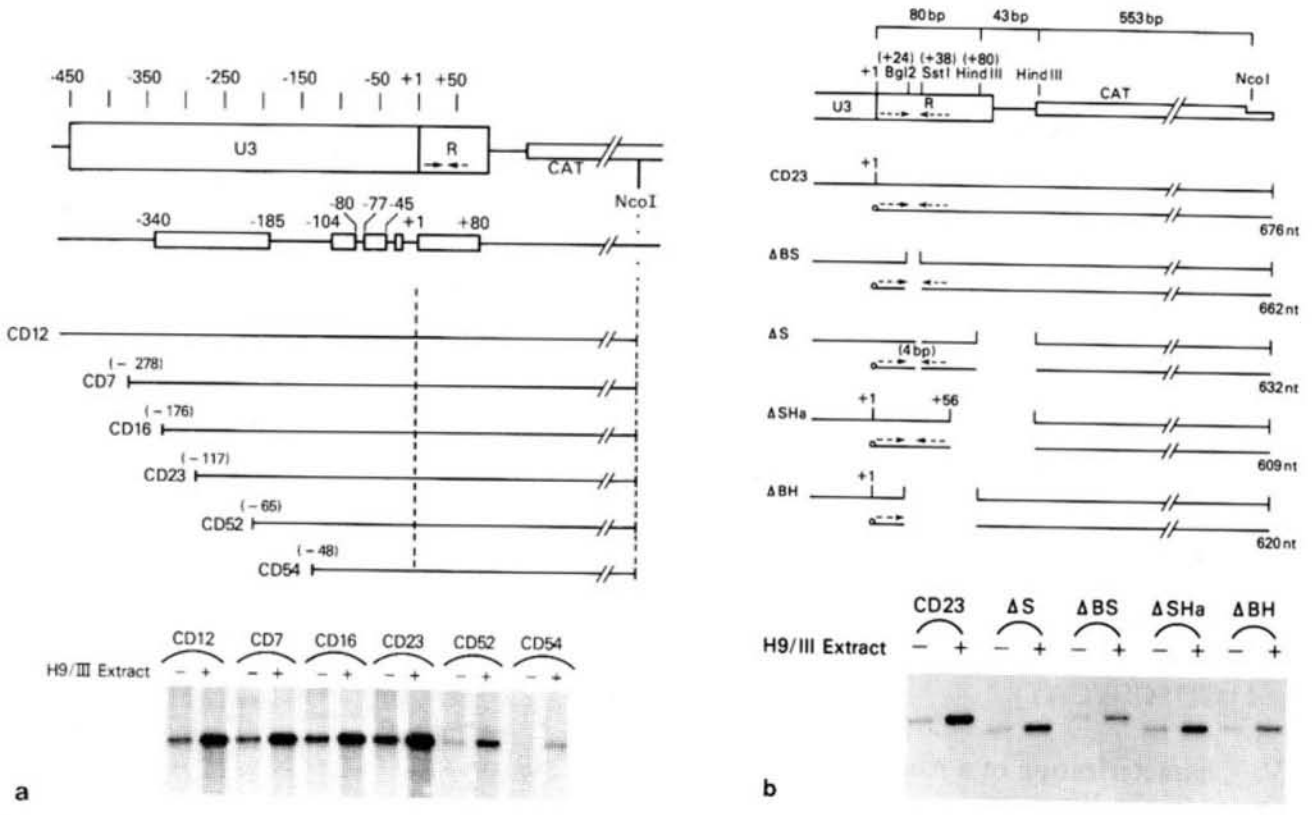

Fig. 2 a, b. 5′ (**a**) and 3′ (**b**) deletion plasmids of pC15CAT. Deletions were made as described in the material and methods section. pCD12CAT (CD12) contained a small deletion at the *Kpn*I site. Each plasmid is numbered corresponding to the distance in nucleotides from the transcriptional start site 1. The 3′ deletion is constructed from plasmid pCD23CAT (CD23) and indicated as ΔBS, ΔS, ΔSHa, and ΔBH. Sizes of the transcripts are labeled in nucleotides (*nt*). Autoradiogramms of the in vitro transcription experiments are shown with (+) or without (−) H9/III extract

tract prepared from HTLV-III infected H9 cells (H9/IIIE). Deletions to position −117 showed gradual enhancement of transcription after incubation with H9/IIIE (Fig. 2, Table 1). Experiments at the post-transcriptional and transcriptional level indicate the presence of an negative regulatory element around the *Kpn*I site and 5′ part of U3.

II. Location of an HTLV-III Enhancer Element

The level of CAT gene expression was substantially decreased with plasmids containing deletions extending to −117 (pCD23CAT). We concluded that the reduced activity might reflect the removal of an EHE, a direct repeated sequence of 10 nucleotides from −104 to −80. If this is the case, substitution would restore functional gene enzyme activity. The data in Table 1 demonstrate indeed that the gene expression was activated after insertion of an EHE independent of orientation in pCD52ECAT or pCD52EWCAT and comparable to pCD12CAT.

III. Importance of Sp1 Binding Sites

Binding of the nuclear transcriptional factor Sp1 has been shown to be specific for GC rich regions [4]. Experiments in which regions of DNA were protected from reagents by specifically bound proteins indicated that the region −77 to −46 contains three tandem, closely spaced Sp1 binding sites of variable affinity (Fig. 1).

To determine if Sp1 binding sites influence the gene expression at transcriptional or post-transcriptional level in combination

with an EHE, we studied this region carefully. The activity of pCD52CAT (−65) was reduced and can be restored after addition of an EHE (pCD52ECAT and pCD52EWCAT). This demonstrates that Sp1 II and I are sufficient for CAT gene expression in the presence of an EHE. However, a deletion to −48 (pCD54CAT) abolishes the activity and substitution of an EHE in sense (pCD54ECAT) or antisense orientation (pCD54EWCAT) restores activity to only 1/3 of the CAT gene expression in comparison to pCD23CAT in infected H9 cell. The in vitro transcriptional results are in agreement with this data. The in vitro transcription of pCD52CAT is dramatically reduced, but can be restored with an enhancer element. The deletion to −48 (pCD54CAT) is inactive irrespective of an EHE (pCD54ECAT and pCD54EWCAT).

IV. Characterization of a Region Responsive to Virus-Associated Trans-Acting Regulatory Factors

Previous studies have shown that heterologous promoter and enhancer sequences could be placed in front of a region from −17 to 80 of the HTLV-III LTR [10]. Specific elements in this region are recognized by factors present in response to virus infection. To determine the location of these elements responsive to *trans*-acting regulatory factors we deleted sequences around a possible hairpin structure (Fig. 3). For these experiments pCD23CAT, a plasmid with high CAT gene activity, was used for all deletions in the R region. A deletion of 54 bp (pCD23ΔBHCAT) inactivates CAT gene activity completely and decreases in vitro transcription (Fig. 2b). pCD23ΔSHCAT, a deletion of 40 bp, shows high promoter activity of Exp2 (Table 1), but CAT expression in infected cells are low in Exp1 and 2. The in vitro transcription is comparable to pCD23ΔSHCAT. Two deletions of 14 bp and 4 bp around the hairpin structure, pCD23ΔBSCAT and pCD23ΔSCAT, are inactive in the CAT gene expression system. The in vitro transcription of pCD23ΔSCAT shows transcripts at decreased level. A deletion of 18 bp between 56 and 84 (pCD23ΔSHaCAT) gave values comparable to pCD23CAT.

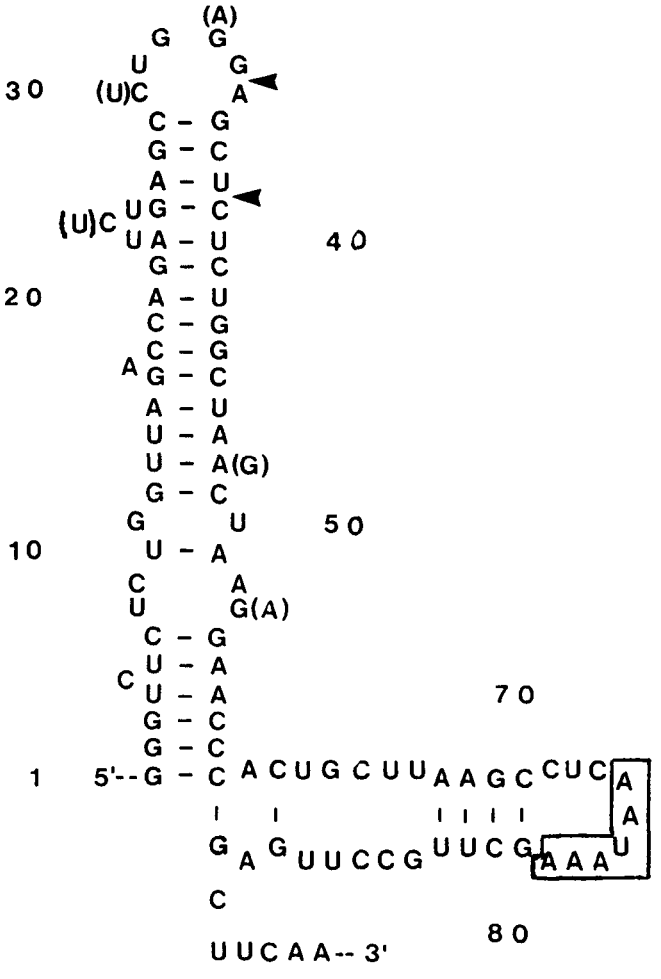

Fig. 3. Possible secondary structure of the nucleotide sequences around the cap site and poly(A) site of C15. In *parentheses* are variation of other virus isolates [8], *arrow* indicates the deletion of four nucleotides (pCD23ΔSCAT). The polyadenylation signal is *boxed*

E. Discussion

The *cis*-acting regulatory elements located within a 160 bp region of the HTLV-III LTR have been described in an in vitro transcription system or CAT gene enzyme expression assay. The presence of the NRE has been demonstrated on the transcriptional and post-transcriptional level by removal of the 5′ part of the 3′ orf and U3 to −117 of pC15CAT. In addition we found differences in CAT gene enzyme response between pC15CAT and pVHHCAT. A explanation for the low activity of pC15 (ca. $^1/_4$ of pCD12CAT) might be a negative regulation of sequences between the *Xho*I and *Kpn*I site. A dramatic loss of transcriptional and CAT enzyme activity upon deletion of sequences between −117 to −65 suggested

the presence of a strong positive-regulatory element. A substitution of the EHE (−104 to −81) restored CAT gene activity and in vitro transcription. However, we can not conclude from this experiment that all of these 24 inserted base pairs are needed for enhancement; one of the 10 bp repeat might be sufficient for *trans*-activation.

Three Sp1 binding sites have been identified from −78 to −45 [4]. Complete loss of transcriptional activity could be attributed to the absence of Sp1 III-I. However, reinsertion of the EHE at −54 (pCD54ECAT and pCD54EWCAT) restored the accurate initiation at the transcriptional level, suggesting that the presence of Sp1 binding sites are not a prerequisite for the accurate initiation of transcription. This findings implicate also that one important component of the virus transcriptional unit interacts with the cellular transcription factor, Sp1, and that this factor must function in conjunction with transcriptional elements located downstream of the RNA initiation site to mediate the response of the LTR to viral *trans*-activation.

Sequences surrounding the site of genomic RNA initiation respond to viral-associated *trans*-acting regulatory factors in the presence of the enhancer. This TRE, including Sp1 II and I, that we have mapped to the region −65 to 56, overlaps the promoter, suggesting *trans*-activation may occur via an increased rate of transcriptional initiation. A deletion to 56 (pCD23ΔSHaCAT) showed transcriptional and CAT gene enzyme activity comparable to pCD23CAT. However, a small deletion of four nucleotides (pCD23ΔSCAT) resulted in an complete loss of CAT gene enzyme expression and remaining in vitro transcriptional activity at decreased level. This observation suggested the possibility that at least part of the increase of the HTLV-III LTR directed gene expression in infected cells might be due to post-transcriptional events, including mRNA stability and transport or initiation of protein synthesis. We can arrange the sequences of TRE into a secondary structure (Fig. 3). If for example pCD23ΔSCAT is configured as proposed, a loss of three dimer linkage binding sites may result in an inactivation of CAT gene expression due to post-transcriptional events, but the activity at the transcription level could be decreased.

References

1. Arya SK, Guo C, Josephs SF, Wong-Staal F (1985) Science 229:69–73
2. Feinberg MB, Jarrett RF, Aldovini A, Gallo RC, Wong-Staal F (1986) Cell 46:807–817
3. Fisher AG et al. (1986) Nature 320:367–371
4. Jones KA, Kadonaga JT, Luciw PA, Tjian R (1986) Science 232:755–759
5. Manley JL, Fire A, Samuels M, Sharp PA (1983) Meth Enzyme 101:568–582
6. Maxam AM, Gilbert W (1977) Proc Natl Acad Sci USA 74:560–564
7. Okamoto T, Wong-Staal F (1986) Cell 47:29–35
8. Ratner L, Chang NT, Gallo RC, Wong-Staal F (1985) Nature 313:277–284
9. Rosen CA, Sodrowski JG, Goh WC, Dayton AI, Lippke J, Haseltine WA (1986) Nature 319:555–559
10. Rosen CA, Sodrowski JG, Haseltine WA (1985) Cell 41:813–823
11. Seigel LJ, Ratner L, Josephs SF, Derse D, Feinberg MB, Reyes GR, O'Brien SJ, Wong-Staal F (1986) Virology 148:226–231
12. Starcich B, Ratner L, Josephs SF, Okamoto T et al. (1985) Science 227:538–540
13. Wong-Staal F, Gallo RC (1985) Nature 317:395–403