PNAAZ-779
C5-336
56049

PROGRESS REPORT
July 1,1987 - December 31, 1987

TITLE: PREPARATION OF A LIVE VACCINE AGAINST THE CAPRINE ARTHRITIS-ENCEPHALITIS VIRUS (CAEV)

Project No. DPE-5544-G-SS-6007-00

INVESTIGATORS: A. YANIV AND A. GAZIT
Department of Human Microbiology
Sackler School of Medicine
Tel-Aviv University
Tel-Aviv, 69978, ISRAEL

For the last six months we have been focusing on several aspects concerning the role of LTR domains on CAEV pathogenesis:

I. Determination of transcriptional activity of CAEV LTR.
Sequence analysis of CAEV genome revealed the presence of transcriptional control elements within CAEV LTR (see Progress Report, July-December, 1986). To establish the transcriptional capability of CAEV LTR, we examined LTR capacity to drive the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene, employing the transient expression CAT assay. To this end we constructed recombinant CAT plasmid, in which the entire CAEV LTR was placed 5' to the bacterial CAT gene. To assay for transcriptional activity, increasing amounts of the recombinant CAT plasmid DNA were introduced into Himalayan tahr ovary cells which are permissive for CAEV replication. CAT activity in the cell extract was measured 48 hours after transfection. As can be seen from the representative assay depicted in Fig. 1, CAEV LTR-CAT plasmid resulted in a high level of CAT activity, which reached a plateau at about 6 μg/8x10 cells (~2 pmole).

II. Functional mapping of LTR domains.

Nucleotide sequence analysis of CAEV LTR detected, in the U3 domain, the presence of sequences conforming to consensus transcriptional promoter sites, as well as several direct repeats that could serve as enhancer elements. In order to functionally identify the regulatory sequences, deletion mutants of the LTR coupled to the bacterial CAT gene, were assessed for their ability to direct CAT activity. Data showed (Fig. 2) that deletion within the U3 region of the CAEV LTR (from nucleotide -186 to -75), plasmid b, causes a 10 fold reduction in the transcriptional activity of CAEV LTR. Furthermore, coupling of this U3 region to an heterologous promoter (SV40 promoter, plasmids f,g; or promoter of equine infectious anemia virus, EIAV, another member of the Lentivirinae, plasmids i,j), augmented CAT expression in a position- and orientation-independent manner. On the other hand, deletion within the R-U5 region (from nucleotides +53 to +168) of CAEV LTR (plasmid c) did not affect LTR transcriptional activity, indicating the existence of a typical enhancer element within the -186 to -75 region of U3 domain of CAEV LTR.

III. Participation of host-limiting factors in the regulation of LTR transcriptional activity.

As can be seen from Fig. 1, increasing amounts of CAEV LTR-CAT plasmid DNA resulted in a linear increase of CAT activity, up to a plateau level that was reached at approximately 2 pmoles of LTR-CAT DNA. This suggests that some cellular factors which probably exist at rate limiting concentrations , sufficient to saturate approximately 2 pmole of CAEV LTR DNA per 8x10⁶ cells, may be involved in controlling LTR activity. To establish the

presence of such host factors , competition assays were conducted, in which increasing amounts of CAEV LTR-CAT DNA were cotransfected into Tahr cells together with an excess molar ratio (x10) of CAEV LTR DNA or pBR322 DNA as a control. As can be seen from Fig. 3, the presence of 5 to 10 molar excess of the complete CAEV LTR domain (U3RU5) caused a decrease (50% conversion as compared to 80% conversion) in the level of CAT activity. On the other hand, the presence of 5 to 10 molar excess of the RU5 domain resulted in an increase (94% conversion as compared to 80% conversion) in the level of CAT activity. These data suggest that the RU5 region carries target structures for negatively acting transcriptional regulatory factors, whereas positively acting regulatory sequences presumably reside in the U3 domain, upstream to the promoter region. Thus, we could conclude that CAEV promoter is regulated by cooperation of positively and negatively acting rate-limiting host factors ,which presumably play a role in the regulation of the intra-cellular levels of virus expression.

IV. Determination of the role of LTR domains in controlling the virus host specificity.

In order to determine the role of LTR sequences in controlling the virus host specificity , various CAT plasmid were constructed (Fig. 4): a plasmid carrying the whole CAEV LTR (U3RU5) linked to CAT gene ; a plasmid carrying the CAEV enhancer-containing U3 linked to SV40 promoter and CAT gene; a plasmid carrying the whole EIAV LTR linked to CAT gene ; a plasmid carrying EIAV enhancer-containing U3 domain linked to SV40 promoter and CAT gene ; SV2 plasmid, carrying SV40 enhancer and promoter ,linked to CAT gene served as a control. The various constructs were introduced into several eukaryotic cell lines - Tahr cells which enable efficient replication of CAEV, canine fetal thymus cells which are permissive for EIAV, but insusceptible to CAEV, as well as several other cell lines such as 3T3/BALB/c cells, human HeLa cell, and hamster CHO cells, all of which do not permit efficient replication of any of the lentiviruses. Generally it can be seen (Fig. 4) that CAEV LTR sequences, are able to promote gene expression in various cell types, regardless of cell capacity to enable efficient viral replication. Moreover, CAEV LTR was found to be much more active (13 to 50 fold) than EIAV in all the tested cell lines, correlating well with the different enhancement capacity of the two viral enhancers. The fact that CAEV LTR was more active than EIAV LTR, even in canine fetal thymus cells, which are permissive only for EIAV replication, suggests that the LTR domain is not the major determinant of the cell tropism of these viruses.
Fig. 4 also demonstrates that CAEV LTR functions differently in each type of cells. Moreover, CAEV enhancer ,when linked to an heterologous promoter is more active in Tahr cells, which are permissive to CAEV replication, than in canine cells, whereas EIAV enhancer, when linked to SV40 promoter is more active in canine cells than in Tahr cells. These results suggest that some cellular factors, which are different in each type of cells, may be involved in controlling LTR activity,in accordance with our

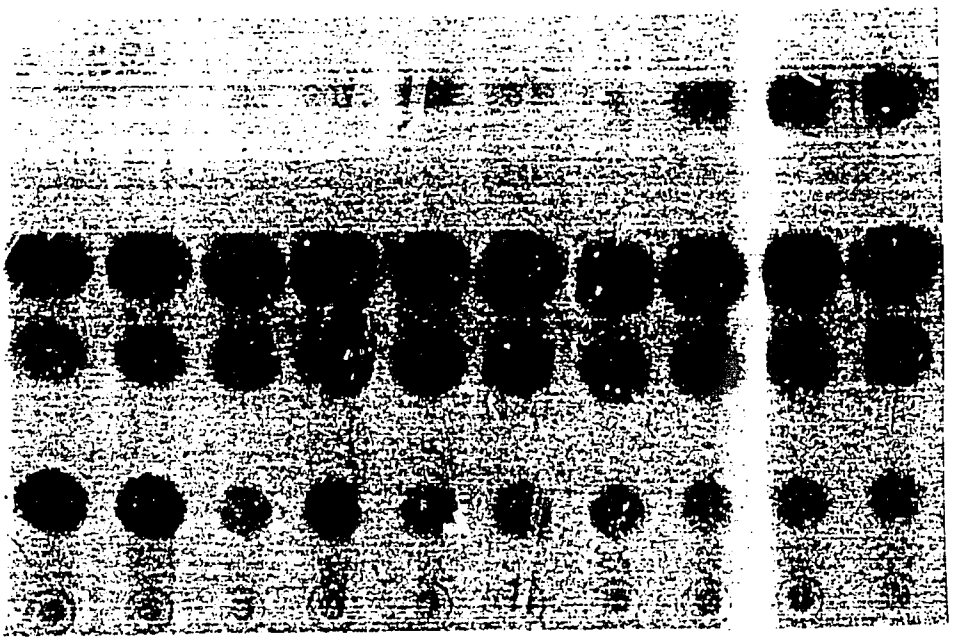
previous data suggesting that some host limiting factors might play a role in regulating viral gene expression.
The contribution of CAEV enhancer to the transcriptional activity of SV40 promoter in each type of cells did not correlate with its contribution when linked to its homologous RU5 region. These data point to the possibility that gene expression in each type of cells is being controlled not only by the enhancer region, but also by other host factors-binding sequences residing in the CAEV RU5 domain.

In addition to the above described experiments we have been continuing our efforts aiming at obtaining an infectious clone of CAEV.

Two main stratagies are being employed:
A. Molecular cloning of CAEV integrated provirus.
High molecular weight DNA, extracted from Himalayan tahr ovary cells, chronically infected with CAEV, was digested with HindIII, which has no restriction site within the viral genome (Yaniv et al, Virology 145, 340 1985). Following size fractionation by sucrose density gradient centrifugation, DNA fragments larger than genome size, were ligated to the HindIII arms of λ 2001 (Stratagene), packaged in vitro and plated onto Escherichia coli strain λ 300 cells, which enable an efficient growth of recombinant phages. Recombinant phages containing CAEV sequences were screened for by in-situ hybridization, using 32P labelled CAEV as a probe. Two recombinant phages were found to contain a full-length genome, boarded by two LTRs. These two clones are now being tested for infectivity.

B. Molecular cloning of CAEV unintegrated form.
As can be judged from the data concerning CAEV LTR activity presented in the present report ,our uninfectious CAEV clone possesses functional LTR domains. Therefore, the stratagy we are employing now , is to use this LTR-containing CAEV subclone as a vector for molecular cloning of the unintegrated form of CAEV into the LTR domains of our non-infectious clone. In this procedure we are trying to replace the EagI-EagI 8.5Kb internal fragment of CAEV of our non-infectious clone (EagI cuts once within each LTR), by the homologous EagI-EagI fragment obtained from the unintegrated CAEV molecules, extracted by the Hirt procedure from freshly infected thar cells. Hundreds of colonies have thusfar been analyzed, without any one being positive.
Since EagI digestion is very inefficient, we are concomitantly employing an additional similar stratagy, in which we are trying to replace the KpnI-KpnI internal fragment (KpnI also cuts once within each LTR) of our non-infectious CAEV clone by KpnI-KpnI fragments obtained from Hirt supernatant. Since KpnI possesses also an internal recognition site within CAEV genome , an additional ligation step will be further required in order to reconstruct a full length CAEV clone.

Fig. No. 1
TRANSCRIPTIONAL ACTIVITY OF CAEV LTR



0.13 0.26 0.52 1.04 2.08

pmoles of
CAEV LTR-CAT
DNA

Increasing amounts of CAEV LTR-CAT DNA were transfected into subconfluent tahr cells. CAT assays were performed on extracts prepared 48 hours after transfection.

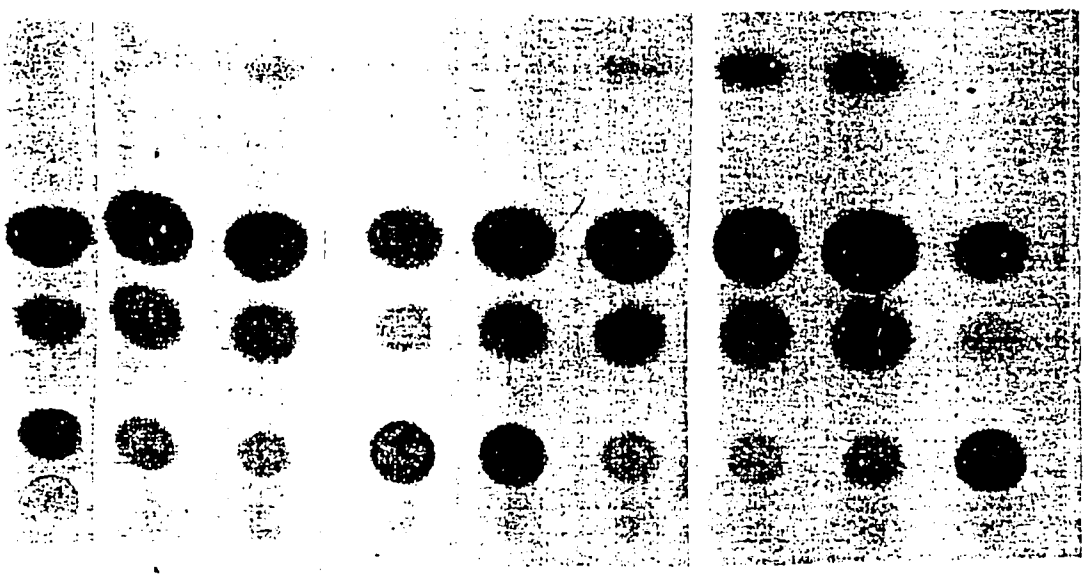
Fig. No. 2
FUNCTIONAL MAPPING OF CAEV LTR DOMAINS

		CONVERSION %	RELATIV ACTIVIT
a.	CAEV LTR-CAT	114	1
b.	CAEV ΔU3RU5-CAT	8	0.07
c.	CAEVU3ΔR-CAT	108	0.9
d.	SV40-Pr-CAT	0.5	1
e.	SV40-En/Pr-CAT	33	66
f.	CAEV-En/SV40-Pr-CAT	44	88
g.	CAEV-En/SV40-Pr-CAT	14	28
h.	EIAV-Pr-CAT	4.5	1
i.	CAEV-En/EIAV-Pr-CAT	72	16
j.	CAEV-En/EIAV-Pr-CAT	73	16

Deletions b, c, were generated from the plasmid CAEV LTR-CAT (plasmid a). Hybrids f, g, were generated by transferring CAEV enhancer region (-183 to -32) to SV2 plasmid, upstream to SV40 promoter, in the positive (f), or negative (g) orientation.
3 μg of the indicated CAT plasmids were transfected into Tahr cells. CAT assays were performed on extracts prepared 48 hours after transfection. CAT activities are expressed as the percentage of the total chloramphenicol convertd to mono acetate products, normalized to 200 μg (80 μl) extract.

Fig. No. 3
PARTICIPATION OF HOST-LIMITING FACTORS IN THE REGULATION OF TRANSCRIPTIONAL ACTIVITY OF CAEV-LTR

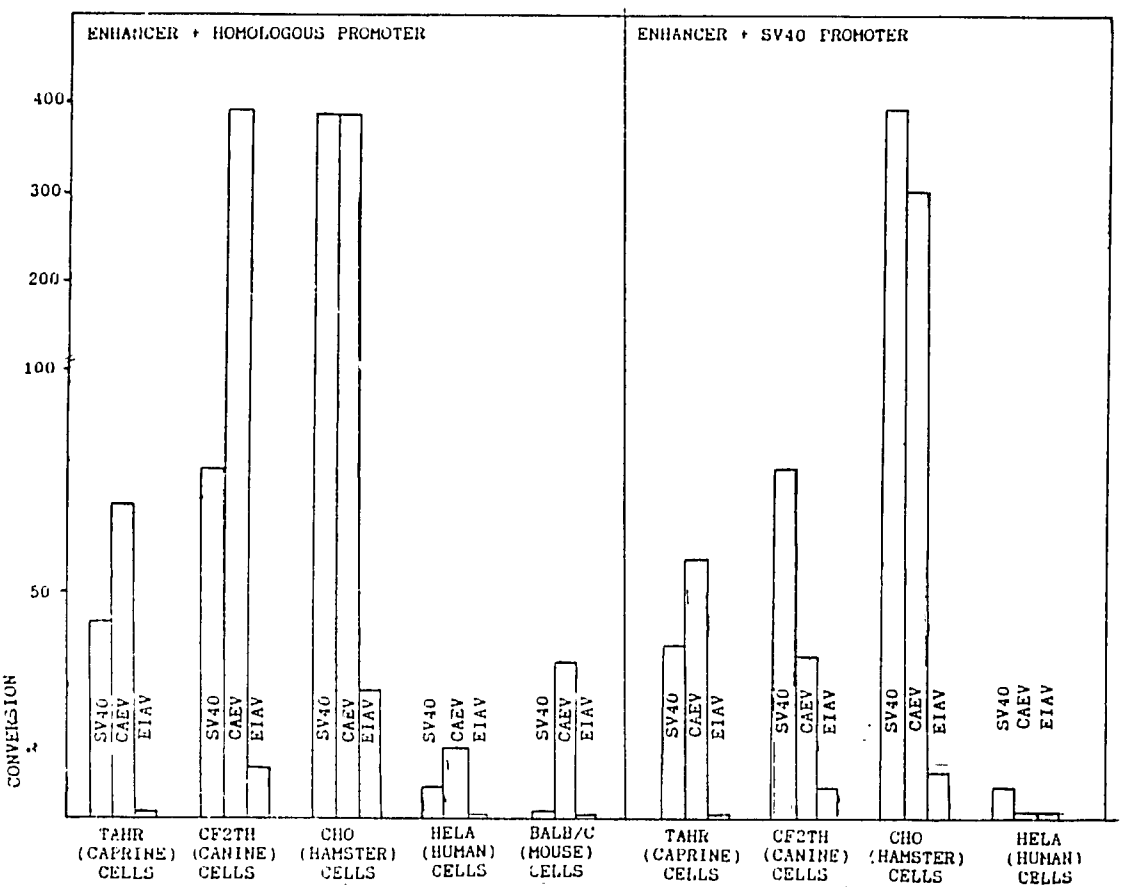
CONVERSION %	80	80	75.4	50	49	85	77	94	92



INDICATOR LTR-CAT (pmoles)	0.13	0.26	0.526	0.13	0.26	0.526	0.13	0.26	0.526
COMPETITOR		pBR322			CAEV-LTR			CAEV-R-U5	

Increasing amounts of LTR-CAT plasmid together with a constant amount (2.6 pmoles, 10 μg, which is 5 to 10 molar excess) of competitor DNAs - the complete CAEV LTR, R-U5 domain of CAEV LTR, and pBR 322 as a control were cotransfected into subconfluent Tahr cells. CAT activity was determined 48 hours after transfection.

Fig. No. 4
THE ROLE OF LTR DOMAINS IN CONTROLLING VIRAL HOST SPECIFICITY



4 µg of the different CAT plasmids were transfected into the indicated cell lines. Enzyme activities were measured after 2 hours incubation with 40 to 400µg of protein used to establish the linear range of the assay. Levels of activity expressed as the percentage of chloramphenicol converted to acetate derivatives, normalized to 400µg protein.