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An Activation-Dependent, T-Lymphocyte-Specific Transcriptional Activator in the Mouse Mammary Tumor Virus *env* Gene

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Transcription of the complete mouse mammary tumor virus (MMTV) proviral genome in mouse cells is controlled by a strong promoter in its long terminal repeat. In the mouse T lymphoma EL4, there is a second, activation-dependent transcriptional initiation site within the envelope (*env*) gene, from which a short mRNA is generated, encoding the open reading frame of the long terminal repeat. We now report the isolation of a segment of the MMTV *env* gene (called META, for MMTV *env* transcriptional activator) which has the expected transcription-activating properties seen in EL4.E1 cells. Namely, it induces activation-dependent, T-lymphocyte-specific transcription of a chloramphenicol acetyltransferase reporter gene. It is active in mouse or human T-helper lymphocyte lines when they are stimulated to transcribe lymphokine genes but is inactive in unstimulated T-helper cells, fibroblasts, a cytotoxic T-lymphocyte line, and a mastocytoma cell line. Its activity is inhibited by cyclosporin A, a specific inhibitor of lymphokine transcription. Several forms of the META have been isolated from EL4.E1 cells, a mouse T-helper cell hybridoma, and from BALB/c spleen cells. Linked to the heterologous thymidine kinase promoter, a 400-bp portion of it is an inducible, orientation-independent, and cyclosporin A-sensitive transcriptional activator in T-helper cells.

Mouse mammary tumor virus (MMTV) is the prototypical B-type retrovirus, whose presence in the milk of nursing mothers of susceptible mouse strains is linked to the development of mammary adenocarcinomas in their female offspring. The MMTV genome does not contain a conventional oncogene, and it is generally accepted that the induction of mammary adenocarcinomas by MMTV is closely tied to the activation of cellular proto-oncogenes by insertional mutagenic activation. The long terminal repeat (LTR) of MMTV can activate nearby genes through steroid hormone-dependent mechanisms. This model predicts that independently generated mammary tumors would often have a provirus inserted near a common gene or genes, which would be abnormally expressed as a result. A search for such genes in mammary tumors has identified several candidate oncogenes.

The development of MMTV-induced mammary tumors in C3H and GR mice is normally restricted to females, as it is estrogen dependent. Male mice of these strains also spontaneously develop tumors, mostly of T-cell origin, although at a somewhat slower rate. Many of these tumors carry amplified numbers of MMTV proviruses, inserted into various genomic sites (10, 30). The acquisition of amplified MMTV provirus copies is characteristic of Thy 1^+, serially transplanted T-lymphoma cells. A common feature of the amplified MMTV in T lymphomas is the occurrence of deletions in the LTR regions of the amplified copies of MMTV (11, 13, 26, 30). These deletions, which vary between about 380 and 500 nucleotides in length, characteristically occur between positions 538 and 1038 of the LTR, whose normal length is 1,337 nucleotides.

The role of MMTV in T lymphomagenesis is generally unproved, but in at least one example, there is a direct relationship. A variant MMTV strain which induces a high incidence of thymic lymphomas in young mice, with high efficiency and short latency, has been isolated (2, 3). This thymotropic variant, DMBA-LV, carries a deletion similar to those found in MMTV proviruses in other T lymphomas, namely, a loss of 440 nucleotides in the central part of U3 in the MMTV LTR. These observations raise the question of whether MMTV also has a particular ability to be expressed in T lymphocytes, perhaps as a function of alterations in the LTR.

A strong connection between MMTV provirus gene expression and lymphocytes is made by the recent discovery that the open reading frame (ORF) found in the LTR is responsible for the minor histocompatibility antigen Mls. MMTV proviruses map to different chromosomal loci in different strains of mice, and the MMTV loci correlate with the expression of different Mls antigens, which, in turn, is correlated with clonal deletions of parts of the T-cell repertoire during ontogeny (1, 8, 12, 15). For example, the LTR ORF found at the Mtv-2 locus has been found to direct the expression of an Mls antigen (1). The likely basis for Mls antigenic differences is the protein sequence variation near the carboxyl end of the ORF protein (8, 36). Although the resulting protein has not yet been found in vivo, it has been synthesized (5, 9), and analysis indicates that it is probably a glycosylated integral membrane protein of 36 kDa. The carboxyl-most 280 amino acids are probably extracellular, with the N-terminal 38 residues forming a cytoplasmic tail which is not essential for Mls antigenicity (9). B lymphocytes express the Mls antigens effectively, but recent evidence indicates that T lymphocytes are more effective at presenting the Mls product as a tolerogen (25, 46). It is as tolerogen that the Mls superantigen has recently received great interest, since the expression of a particular Mls locus dictates the clonal deletion of cells bearing specific T-cell receptor Vβ genes.

It has previously been reported that when certain clonal isolates of the mouse EL4 thymoma cell line are stimulated

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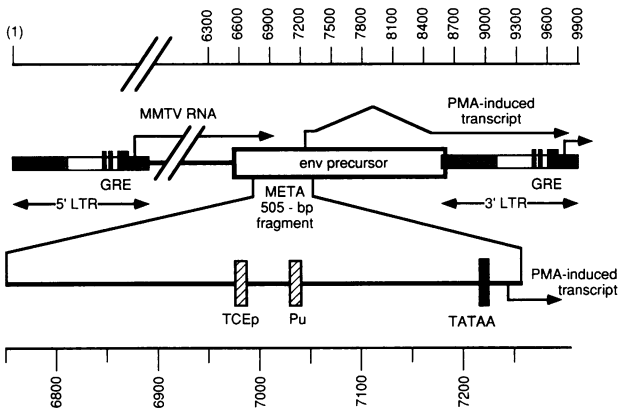

FIG. 1. Relevant features of the MMTV genome. The 3′ portion of the map contains the sequence encoding the *env* protein precursor mRNA and the 3′ LTR. Conventional full-length transcription initiates near the 3′ end of the 5′ LTR (MMTV RNA). In the EL4.E1 T-lymphoma cell line, the LTR (stippled box) contains a 494-bp deletion, shown by the white box within its central portion. This deletion removes a part of the glucocorticoid response elements (GRE). In EL4.E1 cells, the novel promoter in the envelope gene initiates a transcript just downstream of a canonical TATA box and elements which resemble the TCEp and purine (Pu) box transcriptional control sites present in the IL-2 gene (41, 43). The transcript initiated in the *env* gene is spliced as shown (13). The 505-bp segments amplified and cloned from the MMTV *env* transcriptional activator (META) are represented by the fragment indicated.

by phorbol myristate acetate (PMA), a high level of transcription is initiated from the MMTV provirus (13, 24). In contrast to the normal mode of transcription in MMTV and other retroviruses, which initiates within the right-hand part of the 5′ LTR, most of the PMA-induced transcription initiates from within the envelope gene, which lies just upstream of the 3′ LTR of MMTV (Fig. 1). The PMA-induced transcript comprises primarily the 3′ LTR. Induction of this transcription parallels that of several of the cytokine genes in EL4.E1 cells, including interleukin-2 (IL-2), IL-5, and granulocyte macrophage colony-stimulating factor, in that expression is activated by the same inductive signal and inhibited by the immunosuppressive agent cyclosporin A (CsA) (42). This observation suggests that there exists a novel MMTV promoter-enhancer, which is activated in response to signals which induce cytokine transcription in T lymphocytes. This promoter-enhancer generates a transcript which carries the ORF of the MMTV LTR, a sequence which is responsible for the Mls phenotype. In this study, we have isolated a segment of the envelope gene of MMTV proviral DNA from several types of cells and identified a region which contains transcriptional activating sequences which could contribute to T-cell-specific expression.

MATERIALS AND METHODS

Cells and reagents. All cell lines were maintained in RHFM (RPMI 1640, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 100 μM 2-mercaptoethanol, antibiotics, 10% fetal bovine serum). Cytotoxic T-cell lines were maintained in RHFM containing 30 U of human recombinant IL-2 per ml. Cell lines used in the study were S194 (mouse myeloma), P815 (mouse mastocytoma), EL4.E1 (C57BL/6 mouse T lymphoma), MTL2.8.2 (mouse cytotoxic T cells [4]), Jurkat (human T leukemia), LBB (B-cell hybridoma [34]), and 12.1.19 (T-cell hybridoma [14]). Molecular biological reagents and enzymes were obtained from GIBCO BRL Canada (Burlington, Ontario, Canada). PMA, ionomycin, and concanavalin A (ConA) were obtained from Sigma Chemical Co. (St. Louis, Mo.); CsA was a gift from Sandoz Canada Inc. (Dorval, Quebec, Canada). Primers were synthesized in the DNA facility of the Department of Biochemistry, University of Alberta.

Isolation of MMTV fragments. All MMTV sequences are numbered according to the numbering for the milk-borne virus sequence (31). Numbers in parentheses associated with various fragments and oligonucleotides indicate the nucleotide positions of their endpoints. MMTV proviral sequences were isolated from genomic DNA of EL4.E1 cells, 12.1.19 T-hybridoma cells, or primary BALB/c spleen cells by using the polymerase chain reaction (PCR), which was performed with 500 ng of genomic DNA, 0.25 μM each upstream and downstream primer, 200 μM each nucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, and 2.5 U of *Taq* polymerase (Bethesda Research Laboratories) in a total volume of 100 μl. Following denaturation for 3 min at 94°C, samples were cycled 25 times, using 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and a 1.5-min extension at 72°C. The MMTV sequence between 6750 and 9901 (Fig. 1) was copied from DNA of EL4.E1 or 12.1.19 cells by using the upstream (sense) primer Mtv.S02 and the downstream (antisense) primer Mtv.A01 (Table 1). DNA from BALB/c spleen was copied by using the same upstream primer and the downstream primer Mtv.A16, which ends at position 7333. The PCR product from 12.1.19 cells was 3.2 kb in length, whereas the EL4.E1 copy was 494 bp shorter because of the deletion in the LTR (13).

The initial PCR products were cut with the relevant restriction enzymes (i.e., ones embedded in the PCR prim-

TABLE 1. Oligonucleotide primers[a]

Oligonucleotide	Sequence	Restriction site	Position
Mtv.A01	gccgcaTGCCGCAGTCGGCCGACCTGA	*Sph*I	9901
Mtv.A08	CTCTCGGGAGTTCAACCATTTCTG	None	8611
Mtv.A16	GGCGCGGGAAGGCGCAAGGCAACC	None	7333
Mtv.A11	CTTCggaTccATgtcGACCAGTTTTGTATTGGCC	*Bam*HI, *Sal*I	7212
Mtv.S02	GccGAgCTCCGAAGCGGAGGAGGT	*Sac*I, *Sst*I	6750
Mtv.S11	GACCggATccCaaGCTTTGTTGTCTGTCCTCGG	*Bam*HI, *Hin*dIII	6814

[a] The primers sequences are shown 5′ to 3′. They are either sense (Mtv.Sxx) or antisense (Mtv.Axx) relative to the MMTV map. Some of the nucleotides were changed from the known MMTV sequence at their 5′ ends to provide restriction sites (mismatches are indicated in lowercase letters, and restriction sites are underlined). The nucleotide in boldface for each primer corresponds to the MMTV map position given in the last column (numbering of Moore et al. [31]). The sequence of Mtv.A16, used in primer extension, corresponds to the cloned cDNA sequence, which reflects the PMA-induced mRNA found in EL4.E1 sequences (13). It differs slightly from the milk-borne virus sequence at its 5′ end.

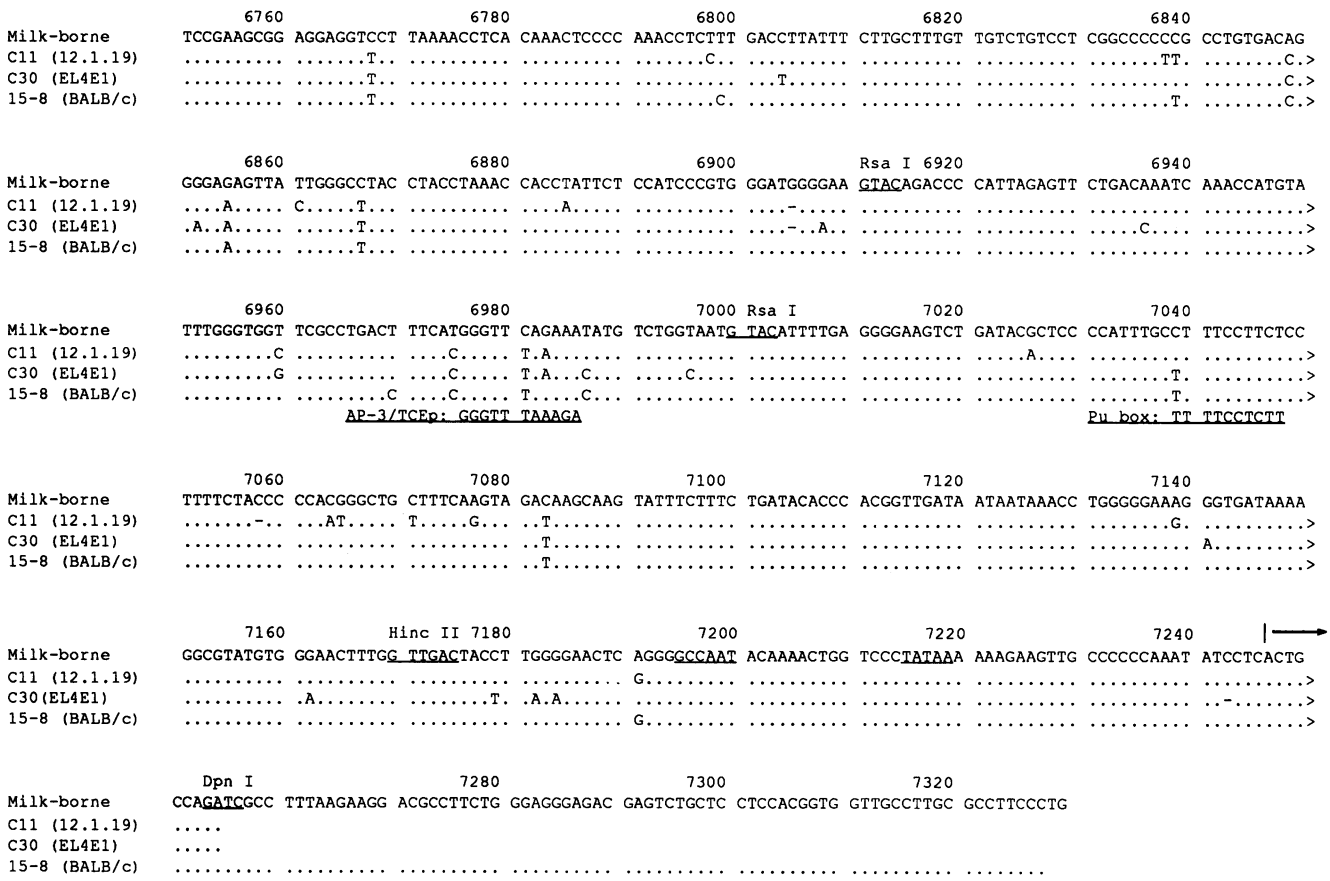

FIG. 2. Sequence comparison between milk-borne MMTV and C11(6750/7255), C30(6750/7255), and 15-8(6750/7333). The milk-borne sequence is numbered as in reference 31. Identity between the sequences of the isolates described in this report and the milk-borne proviral sequence is shown by a dot, and gaps are marked by dashes. The 505-bp META fragments from C30 and C11 were terminated at the *Dpn*I site at 7255, while the 15-8 sequence was terminated at 7333. Transcription of the PMA-induced RNA in EL4.E1 cells initiates at 7247 (see text), 30 nucleotides downstream from the putative TATA box centered at 7217. There is a CCAAT sequence at 7196. The motifs marked AP-3/TCEp and Pu (purine) box are found in the upstream regulatory region of IL-2 (see Discussion).

ers; Table 1) and cloned in plasmid pGEM3Z (Promega Corp.) which had been cut with *Sst*I and *Sph*I. After cloning, *env* fragments covering the MMTV sequence from 6750 to 7255 were excised from the pGEM3Z recombinant with *Eco*RI (which lies just to the 5′ side of the *Sst*I site in pGEM3Z) and *Dpn*I (MMTV sequence position 7255; Fig. 2). The fragments were treated with Klenow DNA polymerase to fill in the *Eco*RI sites and blunt-end ligated into the *Sma*I site of pGEM1(cat) or pGEM2(cat) (17). The LTR segments were excised from the pGEM3Z recombinants with *Pst*I (MMTV position 8586) and *Hin*dIII (in the pGEM3Z multiple cloning site) and cloned into pGEM2(cat) which had been treated with the same restriction enzymes. The pGEM1(cat) and pGEM2(cat) vectors were constructed by ligation of the 1.6-kb *Hin*dIII-*Bam*HI fragment from pSV2(cat) (22), which includes the bacterial chloramphenicol acyltransferase (CAT) gene and the small-T intron and polyadenylation signal from simian virus 40, into the *Pvu*II sites of pGEM1 and pGEM2 (Promega), respectively (17). Figure 3 shows a schematic depiction of the pGEM2(cat)-MMTV recombinant plasmids. The MMTV fragment copied from BALB/c splenic DNA differed slightly from the others in that it extended from positions 6750 to 7333. It was inserted into pGEM2(cat) DNA which had been cut with *Sst*I and *Sma*I. Shorter fragments of the MMTV region were also introduced into the pGEM2(cat) vector. These were generated by cleaving the original 505-bp inserts with either *Rsa*I or *Hin*cII (cleavage at position 7002 or 7172, respectively).

Shorter segments of MMTV were generated for insertion into the vector pBLCAT2, which contains the herpes simplex virus thymidine kinase (*tk*) promoter upstream of the CAT coding sequence (28). These were copied from the primary MMTV clones by copying with sense primer Mtv.S11 (position 6814) and antisense primer Mtv.A11. This antisense primer begins copying the MMTV sequence at 7212 and deletes the TATA box and the starting point for transcription which occur downstream from it. This PCR product was cleaved with *Bam*HI and inserted into pBLCAT2 which had been treated with the same restriction enzyme. Recombinants were screened to determine the orientation of the MMTV fragment.

Plasmid pCM1 (Fig. 3) was created to generate the RNA probe used for RNase protection experiments. Plasmid pBLCAT2-C30(7212/6814) was cut within the multiple cloning site with *Sph*I and within the CAT coding region with *Pvu*II. The resulting fragment, approximately 720 bp long, was ligated into pGEM3Z (Promega) which had been digested with *Sph*I and *Sma*I. The resulting plasmid contained the MMTV sequence between positions 6814 and 7212, in the reverse orientation, upstream of the 156-bp *tk* promoter,

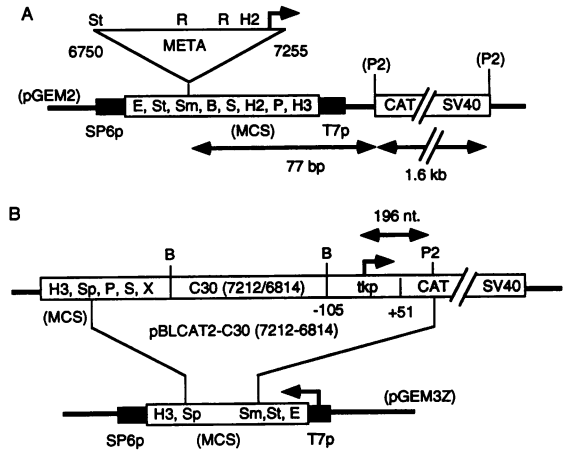

FIG. 3. Plasmid constructs. (A) General structure of the pGEM2(cat) plasmids into which various META fragments were introduced. The arrow in the META fragment designates the transcriptional start site found in stimulated EL4.E1 cells (Fig. 4), which is also indicated in Fig. 1. The coding sequence of the CAT gene was 77 bp from the insertion site of the META fragments. The 1.6-kb fragment containing the CAT coding region and simian virus 40 (SV40) splice and polyadenylation regions is inserted into the *Pvu*II site of pGEM2 as described in Materials and Methods. (B) Structures of pBLCAT2-C30(7212-6814) and pCM1. The META fragment in this case ends upstream of the TATA box (position 7215; Fig. 2) and is in the reverse orientation relative to the MMTV map. The starting site for transcription within the *tk* promoter (tkp) is indicated and lies 51 bp upstream of the starting point of the CAT coding sequence. The segment indicated was transferred to pGEM3Z, in which T7 RNA polymerase was used to transcribe it. Only 196 nucleotides of the resulting RNA probe should be protected by transcripts which initiate in the *tk* promoter. Restriction sites: E, *Eco*RI; B, *Bam*HI; H2, *Hin*dII; H3, *Hin*dIII; P, *Pst*I; P2, *Pvu*II; R, *Rsa*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; St, *Sst*I-*Sac*I; X, *Xba*I. Also indicated are the multiple cloning site (MCS) and the T7p, SP6p-T7, and SP6 promoters.

which in turn was followed by the 5′ end of the CAT coding sequence (Fig. 3).

Plasmid pRSV(cat) (21) was used as a constitutively active strong promoter construct.

Primer extension. Total cellular RNA was isolated by the guanidinium thiocyanate-cesium chloride gradient method (7). T4 kinase was used to end label oligonucleotide primers Mtv.A08 and Mtv.A16 (Table 1) with ^{32}P. The primer extension assay was performed essentially as described elsewhere (40). Briefly, 10 μg of total RNA was hybridized overnight at 45°C with 5×10^4 cpm of labeled oligonucleotide probe (2.4×10^7 cpm/μg). Reverse transcription was done at 42°C with cloned Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). Products were separated under denaturing conditions on a 7 M urea–6% acrylamide gel and visualized by autoradiography. Labeled, denatured fragments generated from pGEM2 DNA cut with *Hin*fI, and PM2 DNA cut with *Hae*III, were used as markers.

RNase protection assay. Plasmid pCM1 was linearized with *Sal*I, and radiolabeled RNA was synthesized by using [α-^{32}P]CTP (800 Ci/mmol), T7 RNA polymerase, and the Promega Riboprobe system. The RNase protection assay was performed according to the protocol described by Promega. Total RNA isolated from the transfected, unstimulated, or stimulated cells (50 μg) was hybridized in 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–0.4 M NaCl–1 mM EDTA with 5×10^5 cpm of RNA probe for 6 h at 45°C. Nonhybridized probe was digested with 40 μg of RNase A per ml for 1 h at 30°C. Following extraction with phenol-chloroform and ethanol precipitation, the protected RNA fragments were analyzed in a 6% polyacrylamide–7 M urea gel and then subjected to autoradiography.

Transfection procedure and CAT assay. Plasmid DNA for transfection was purified by CsCl gradient centrifugation as required and was greater than 90% closed circular DNA, as assayed by ethidium bromide fluorimetry (32). Cells were transfected by a modified DEAE-dextran procedure (17). Cells in log growth phase were washed twice in serum-free medium and resuspended in Tris-buffered saline (25 mM Tris-HCl [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$); 1.5×10^7 cells in Tris-buffered saline were added to 500 μg of DEAE-dextran per ml and 15 μg of plasmid DNA in a final volume of 2 ml. After 30 min at room temperature, the cells were washed in RHFM and recultured at 2×10^5/ml under the conditions and for the times indicated in individual experiments. At harvest, cells were washed once in phosphate-buffered saline and resuspended in 0.1 ml of 0.25 M Tris-HCl (pH 7.5). After three freeze-thaw cycles, the preparation was spun for 5 min at 14,000 rpm, and the supernatant was stored at −70°C. Protein concentration was determined with a Coomassie protein assay kit (Bio-Rad), and the same amount of protein was used for all samples within a given experiment. Up to 70 μl of cell extract was incubated with 0.02 μCi of [^{14}C]chloramphenicol (New England Nuclear), 0.53 mM acetyl coenzyme A, and 0.25 M Tris-HCl (pH 7.5) in a total of 150 μl for 4 h at 37°C. Acetylated products were analyzed by thin-layer chromatography and autoradiography and quantitated by liquid scintillation counting as described elsewhere (17).

RESULTS

Transcription initiation within the MMTV *env* gene. A partial map of the MMTV genome is shown in Fig. 1. Conventional transcription initiates within the 5′ LTR and generates a full-length primary RNA molecule. However, in the T-lymphoma cell line EL4.E1, there appears to be a strong PMA-inducible promoter within the *env* gene (13), which we term the MMTV envelope transcriptional activator (META). The primary transcript which is generated contains the remainder of the *env* precursor region and the 3′ LTR, and it is spliced to remove most of the *env* component (Fig. 1). The splice acceptor site is the same one used in the generation of the so-called LTR transcript from the conventionally transcribed, full-length MMTV RNA (31). On the basis of sequence analysis and 5′-end mapping, we determined that the transcript appears to initiate about 30 nucleotides downstream from a consensus TATA box element (13).

To confirm the start site for this transcript and show that this segment of the *env* gene is a promoter in intact cells, MMTV RNA from induced EL4.E1 cells was examined by the primer extension technique. Two EL4.E1 subclones were selected on the basis of their capacity to produce high levels of IL-2 in response to PMA. Total cellular RNA was isolated from unstimulated EL4.E1 cells, from cells treated for 12 h with 15 ng of PMA per ml, or from cells treated with PMA and 100 ng of CsA per ml. CsA blocks the transcription of IL-2 and also the accumulation of the PMA-induced MMTV transcript. The oligonucleotide probe Mtv.A08,

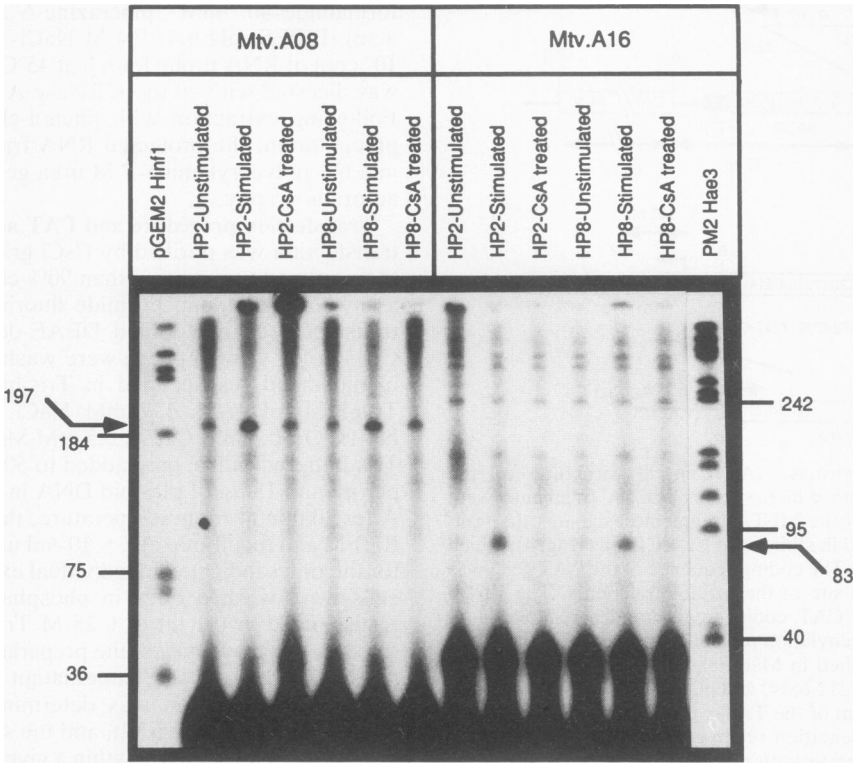

FIG. 4. Primer extension assay of MMTV mRNA induced in EL4.E1 cells by PMA. Total cellular RNA was isolated from two EL4.E1 clones, HP2 and HP8, which were either untreated or treated with PMA (15 ng/ml) in the presence or absence of CsA (100 ng/ml) for 12 h. Ten micrograms of RNA was used for each primer extension reaction. Primer Mtv.A08 begins at position 8611 of the MMTV sequence, and Mtv.A16 begins at position 7333 (Table 1). The positions of markers (*Hae*III-digested PM2 and *Hin*fI-digested pGEM2 DNA fragments) are shown. Arrows indicate the predominant primer extension products and their lengths in nucleotides, as determined by the results of three experiments.

which has its 5′ end at nucleotide 8611, was designed to detect the spliced transcript, as it is positioned to the right of the intron splice junction. A second oligonucleotide, Mtv.A16, primes to the 5′ side of the intron, at position 7333, and detects both spliced and unspliced transcripts.

The results are shown in Fig. 4. The major product with primer Mtv.A08 was approximately 197 nucleotides long, while the Mtv.A16 product was 83 nucleotides long. These products either were present at low levels or were undetectable in uninduced cells, and the increase induced by PMA was suppressed by CsA, corresponding to the pattern found earlier for the 13S mRNA of MMTV (13). The predicted starting points of the mRNA are 7253 and 7250, respectively. Sequence analysis of cDNA clones (13) indicated that the first nucleotide found in the mRNA was the A residue at 7247, so this is taken as the most likely 5′ end of the PMA-induced mRNA. The results with primer Mtv.A16, which primes to the left of the 1,161-nucleotide-long intron found in the 13S mRNA, clearly indicate that the starting point is at or near residue 7247. Those obtained with primer Mtv.A08 show a predominant product which corresponds to the same initiation site for the RNA, if the intron is spliced out. There may, however, be additional molecules in which the intron has not been removed, which would produce material not resolved by this gel analysis.

In summary, an MMTV transcript is initiated within the *env* gene of MMTV provirus(es) of EL4.E1 cells, and its expression is controlled by the same signals which induce or suppress cytokine transcription in these cells. These results raise the question to which the remainder of the results are directed, namely, whether this transcription is activated by elements within the MMTV *env* gene, as distinct from more distal regulatory elements such as those in the LTR, and if so, whether such elements respond to activation signals in T lymphocytes.

Isolation of MMTV proviral sequences. EL4.E1 cells, in addition to having three germ line MMTV proviruses, also have amplified provirus copies which contain within their LTR a 494-bp deletion (13, 24, 30), and it is these deletion versions which are the template for PMA-induced transcription (13). To study the META sequences in the MMTV provirus, clones of PCR products of the right-hand end of the MMTV provirus, extending between nucleotides 6750 and 9901, were generated. The cloned sequences C30 and C32 were derived from EL4.E1 DNA. C11 was from 12.1.19 cellular DNA, and 15.8 was from BALB/c spleen cells. The 12.1.19 T-helper lymphocyte cell line secretes IL-2 in response to either ConA, anti-CD3ε antibody, or its nominal antigen, poly-18 (14), but Northern (RNA) blot analysis and primer extension indicated that it does not express the short, META-controlled transcript found in activated EL4.E1 cells (results not shown). Sequence analysis confirmed that the EL4.E1 DNA clones contained the 494-bp deletion of the amplified provirus forms. Unexpectedly, the 3′ LTR of the 12.1.19 clone contained a substitution, in which the region from 9462 to 9552 was replaced by 107 bp of unknown origin. A 505-bp region of the *env* gene from each of the EL4.E1 and 12.1.19 isolates was cloned into pGEM2(cat), as were the

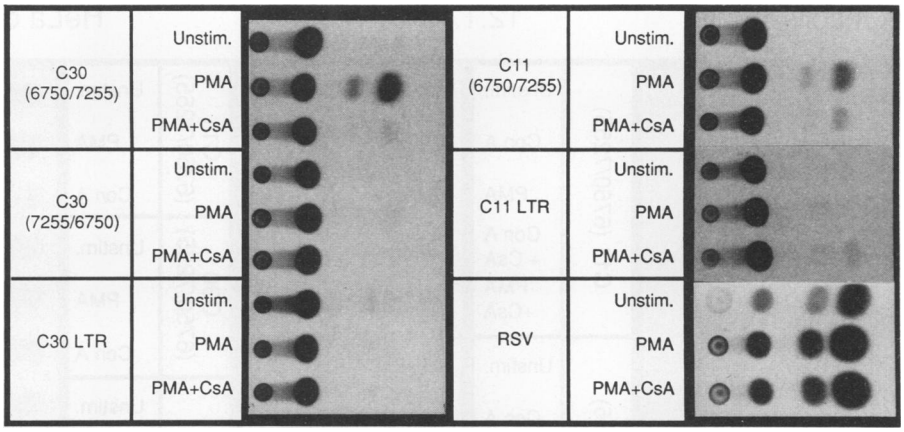

FIG. 5. Induction of CAT activity in EL4.E1 cells by PMA and suppression by CsA. EL4.E1 cells were transfected with META-containing constructs from two different clones (C30 and C11), the LTR fragments from the same clones, or C30 in the reverse orientation (7255/6750), all attached to a promoterless CAT gene. C30 was derived from EL4.E1 cells, and C11 was derived from 12.1.19 T hybridoma cells. The positive control is plasmid pRSV(cat) (21). Cells were either unstimulated or stimulated with PMA (15 ng/ml) in the presence or absence of CsA (100 ng/ml) for the final 15 h of a 42-h incubation period following transfection.

corresponding LTR sequences. The region from 6750 to 7333 of BALB/c spleen cell MMTV was also inserted into the CAT expression plasmids. BALB/c mice, although not a high-incidence tumor strain, can develop both mammary tumors and lymphomas. The sequences of the cloned *env* fragments of C11, C30, and 15.8 are compared with that of the milk-borne virus in Fig. 2 (see also Discussion).

Transient expression of CAT constructs. To determine transcription-activating functions of the MMTV *env* region fragments, plasmid DNAs containing the various segments were used to transiently transfect EL4.E1 cells by the DEAE-dextran procedure. As shown in Fig. 5, the 505-bp *env* fragment derived from clone C30, extending from MMTV positions 6750 to 7255 and designated C30(6750/7255), and the comparable fragment from clone C11 activated CAT gene expression in transfected cells which were stimulated with PMA but not in unstimulated cells. This transcriptional activity was blocked by CsA. C30 in the reverse orientation, designated C30(7255/6750), was inactive, as were the corresponding C30 and C11 3′-LTR fragments. The LTR fragments contain glucocorticoid response elements, which require glucocorticoids and their receptors for activity, neither of which was present (EL4.E1 cells appear to lack glucocorticoid receptors). The control plasmid containing a Rous sarcoma virus (RSV) promoter-enhancer was highly active under all conditions, as expected. Although several different cloned META sequences have been obtained from EL4.E1 cells, some versions of the same region do not support expression of the CAT gene (data not shown). The basis for this difference presumably lies in the sequence differences which were shown to exist between various clones (Fig. 2 and unpublished data).

Cell specificity of expression. The transcriptional activating properties of the META-CAT constructs were tested in several types of cells (Fig. 6). C11(6750/7255) supported inducible CAT gene expression in Jurkat cells (human T-leukemia cell line) and in the mouse T hybridoma 12.1.19 but not in HeLa cells. A second EL4.E1-derived fragment, C32(6750/7255), showed the same pattern of activation in 12.1.19 cells. The requirements for induction of C11(6750/7255) in Jurkat cells were the same as those for induction of the IL-2 gene in these cells; namely, ConA and PMA alone were weak inducers but synergized to yield strong induction. CsA blocked the induction of CAT activity in both Jurkat and 12.1.19 cells, as has also been found for the IL-2 gene (42). The RSV promoter-enhancer was highly active in all of these cell lines, independent of cellular stimulation or suppression.

As yet we have been unable to detect an appreciable level of CAT activity in several other types of cell lines which were transfected with C11 or C30 constructs (Table 2). We detected a low level of activity (twofold over untreated cells) in LBB 3.4.16 cells treated with PMA. This B-cell line is known to express the MMTV-directed Mls^a antigen (34). No activity was detectable in S194 B cells or in the P815 mastocytoma cell line, HeLa cells, or a cytotoxic T-cell line.

The T-hybridoma cell line 12.1.19 can be induced to synthesize IL-2 with ConA, or its nominal antigen, or anti-CD3ε antibody but not with PMA. As shown in Fig. 6, ConA induced CAT expression by C32(6750/7255) and C11(6750/7255) in 12.1.19 cells, whereas PMA alone had no effect. Figure 7 demonstrates that anti-CD3ε antibody can also induce transcriptional activation by the C30 and C11 constructs containing the 505-bp META fragment. The corresponding LTR fragments were nonfunctional under the same conditions. These results suggest that the 6750/7255 META fragments are capable of supporting transcription only in T helper cells, and then only following activation by the same stimuli which induce cytokine production in these cells.

Localization of transcription-activating sequences. A series of fragments was generated by using available restriction sites within the 6750/7255 region of the C30 MMTV sequence. Subfragments were cloned into the pGEM2(cat) vector and used to transfect Jurkat cells. ConA and PMA, or ionomycin and PMA, were added for the final 12 h of a 42-h incubation period. The full-length 505-bp fragments from isolates C30 and C11 and from clone 15-8 were included for comparison. As shown in Table 3, 15-8(6750/7333) induced CAT gene expression in stimulated cells by over 100-fold in comparison with untreated cells, but the induced expression was only moderately sensitive to CsA. Induction of transcription with the full-length constructs C11(6750/7255) and C30(6750/7255) was also strong, yielding 103- to 260-fold

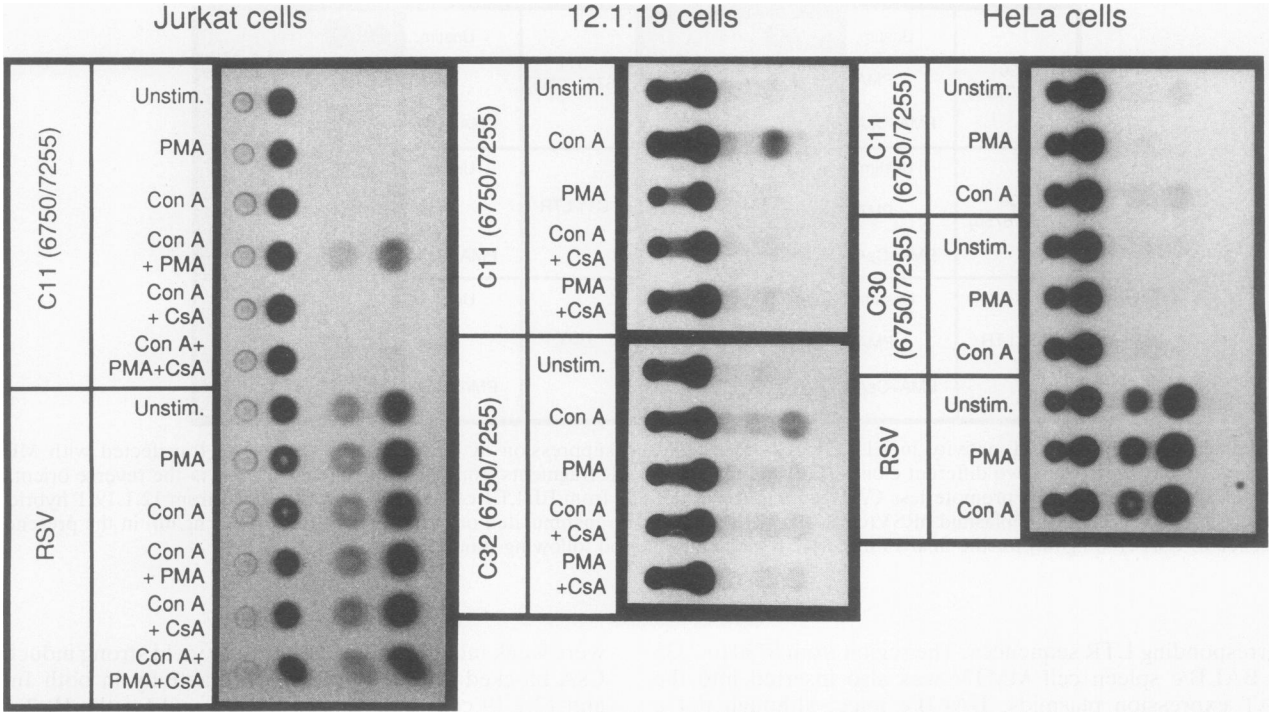

FIG. 6. Specificity of META function. The indicated constructs were transfected in Jurkat (human T leukemia), 12.1.19 (mouse T-cell hybridoma), or HeLa (human epithelioid carcinoma) cells. Jurkat cells were treated with 10 ng of PMA per ml and/or 30 μg of ConA per ml; 12.1.19 and HeLa cells were treated with 15 ng of PMA per ml or 10 μg of ConA per ml. CsA was used at 100 ng/ml. An equivalent amount of protein was assayed for each cell type except 12.1.19 cells treated with ConA, for which one-half the amount was used. C32 is another cloned MMTV sequence derived from EL4.E1 cells.

enhancements, depending on experimental conditions. C30(6750/7255) showed the greatest sensitivity to CsA. CAT activity generated from pRSV(cat) was similar under all circumstances. The construct C30(6750/7172), which does not contain the *env* TATA box, did not support transcription. C30(7173/7255), although containing the TATAA sequence, was also inactive. A C30 fragment which extends from 7002 to 7255 did enhance transcription 18-fold over the level detectable in uninduced cells. This region contains a purine-rich segment which is almost identical to the core of the NF-AT binding sequence in the human and mouse IL-2 genes (41, 43) (see Discussion). These results suggest that although the region extending from 7002 to 7255 within the C30 clone derived from EL4.E1 cells is partially active in stimulated cells, and this activity is suppressible by CsA, sequences 5′ to this region are required for full transcriptional activation.

Although the loss of expression upon deletion of the segment between 7173 and 7255 from clone 30 is consistent with the notion that the TATA box and mRNA start site in the META sequence (nucleotides 7215 to 7246) form the site of transcriptional initiation in the recombinant CAT con-

TABLE 2. Transcriptional activation by the 505-bp MMTV envelope segment in various cell lines[a]

Cell type	Activation in stimulated cells (agent)
T-helper cell lines	
EL4.E1	+ (PMA)
Jurkat	+ (PMA + ConA)
12.1.19 hybridoma	+ (ConA)
12.1.19 hybridoma	+ (anti-CD3ε)
B-cell lines	
S194	− (PMA or ConA)
LBB 3.4.16	− (LPS)
LBB 3.4.16	+/− (PMA)
Other	
P815 (mastocytoma)	− (PMA or ConA)
HeLa (endothelial carcinoma)	− (PMA or ConA)
MTL2.8.2 (cytotoxic T cell)	− (PMA or ConA)

[a] The indicated cell lines were transfected with the C30(6750/7255) or C11(6750/7255) construct in pGEM2(cat), and the indicated stimulating agents were added for the final 15 h of culture. Results were graded on whether CAT activity obtained within any transfection group was greater than that obtained with mock-transfected cells (no plasmid DNA present). Less than a twofold increase was considered insignificant. Unstimulated cells showed no CAT activity. LPS, lipopolysaccharide.

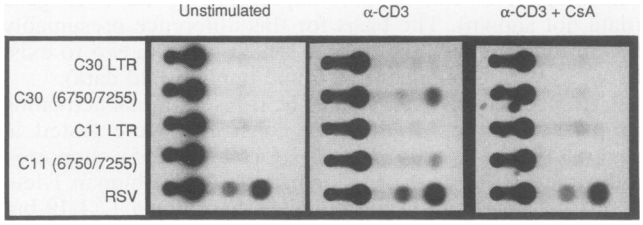

FIG. 7. Induction of META in a T-cell hybridoma by anti-CD3ε (α-CD3ε). Cells of the T-helper hybridoma line 12.1.19 were transfected with the C30 or C11-derived META or LTR constructs and treated with a monoclonal antibody to CD3ε (27) in the presence or absence of 100 ng of CsA per ml during the final 12 h of incubation.

TABLE 3. Activation of CAT transcription by segments of the MMTV envelope gene[a]

Expt no.	Promoter-enhancer	Activation[b]		
		Untreated	Treated	Treated + CsA
1	pRSV	88	91 (1.0)	90 (1.0)
	15-8(6750/7333)	0.7	88 (122)	69 (96)
	C11(6750/7255)	0.3	81 (260)	25 (80)
	C30(6750/7255)	0.3	42 (140)	1.1 (3.3)
	C30(7002/7255)	0.5	9 (18)	0.9 (2)
	C30(7173/7255)	0.4	0.3 (1.0)	0.4 (1.0)
	C30(6750/7172)	0.3	0.4 (1.0)	0.3 (1.0)
2	pRSV	48	58 (1.2)	52 (1.1)
	15-8(6750/7333)	0.55	74 (135)	11.8 (21)
	C11(6750/7255)	0.5	54 (109)	21 (42)
	C30(6750/7255)	0.4	41 (103)	0.48 (1.2)

[a] Jurkat cells were transfected with pGEM2(cat) plasmid DNA containing the MMTV segments indicated (see Fig. 1 for numbering). In experiment 1, cells were treated with PMA (10 ng/ml) and ConA (30 μg/ml), without or with CsA (100 ng/ml) for the final 12 h of a 42-h culture period. Each CAT assay contained 350 μg of protein. In experiment 2, cells were treated with PMA (15 ng/ml) plus ionomycin (1.5 μM), and 250 μg of protein was used per CAT assay. pRSV(cat) was used as a constitutive, CsA-insensitive control.

[b] Expressed as the percentage of total substrate acetylated, with the numbers in parentheses indicating activity relative to that obtained with untreated cells.

structs, this possibility is not proven. It is also possible that sequences within the pGEM(cat) plasmids, lying between the META fragment and the CAT coding sequence (Fig. 3), act as transcription initiation sites. The question of transcriptional initiation is addressed directly for a related construct which uses the *tk* promoter (Fig. 8 and 9).

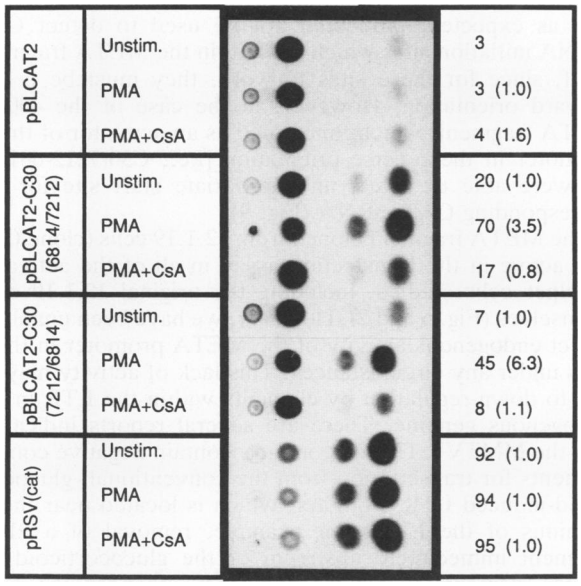

FIG. 8. Evidence that the MMTV *env* gene fragment between positions 6801 and 7212 is an orientation-independent, CsA-sensitive transcriptional activator. C30(6801/7212) (forward orientation) and C30(7212/6801) (reverse orientation) were inserted into pBLCAT2 and used to transfect EL4.E1 cells. Cells were either untreated or treated with PMA (15 ng/ml) or with PMA and CsA (100 ng/ml) during the final 15 h of a 42-h incubation period. The percentage of acetylated product is shown at the right, with numbers in parentheses indicating the level of activity relative to that obtained with untreated cells.

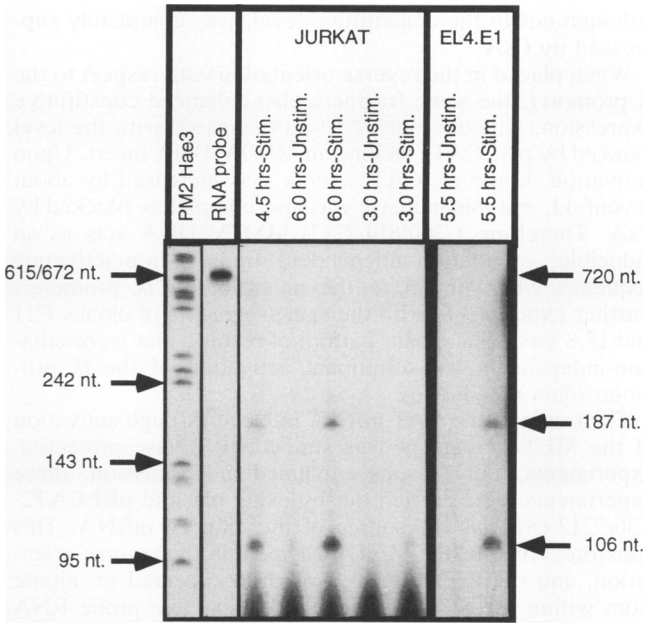

FIG. 9. Start site of the mRNA generated from a pBLCAT2-META construct. EL4.E1 HP2 cells and Jurkat cells were transfected with pBLCAT2-C30(7212/6814). Following incubation for 20 h cells were treated with 15 ng of PMA per ml and 1.5 μM ionomycin for the times indicated. Total RNA was isolated and subjected to the RNase protection assay. The RNA probe was produced from plasmid pCM1 (Fig. 3) and was approximately 720 nucleotides (nt) in length (right-hand lane). Transcripts initiating from the *tk* promoter should be 196 nucleotides long, essentially the same as the longer of the two protected fragments shown in the figure. The shorter protected fragment, 103 to 106 nucleotides long, likely corresponds to probe-RNA hybrids which were cleaved within an A-rich region present between positions 7 and 13 with respect to the translational start site of the CAT coding region. This artifactual cleavage has been described elsewhere (20). Note that both the longer and shorter fragments were increased in stimulated cells. The markers were derived by ^{32}P labeling PM2 DNA which had been cut with *Hae*III.

Activation of a heterologous promoter. Experiments were carried out to determine whether the cloned META sequences contain elements which can control the activity of a heterologous promoter. We used the vector pBLCAT2, which contains the herpes simplex virus *tk* promoter upstream of the CAT coding sequence. To remove any contribution or interference by the nominal start site within the *env* promoter, the C30 fragment was copied with PCR primers Mtv.A11 and Mtv.S11 (Table 1). The antisense primer replaced the TATA sequence and copied only the sequence to the left (upstream) of it, thus removing the transcriptional start site at 7247. The copied fragment, containing MMTV sequences from 6814 to 7212, was shorter than C30(6750/7255) by 64 bp at the 5′ end and 43 bp at the 3′ end.

Recombinant plasmids were transfected into EL4.E1 cells, which were treated with PMA or with PMA and CsA for the final 12 h of culture (Fig. 8). The *tk* promoter alone (pBLCAT2 vector) induced a low but detectable level of CAT expression, but this activity was insensitive to activation by PMA or suppression by CsA. Insertion of the META-derived fragment C30(6814/7212) upstream of the *tk* promoter increased the level of CAT expression by about 7-fold even in unstimulated cells and was further increased 3.5-fold upon activation with PMA. The inducible increase,

although not to the constitutive level, was completely suppressed by CsA.

When placed in the reverse orientation with respect to the *tk* promoter, the same fragment also enhanced constitutive expression, although only 2.3-fold compared with the level induced by pBLCAT2 lacking an MMTV DNA insert. Upon activation, however, CAT activity was increased by about sevenfold, and this increase was also completely blocked by CsA. Therefore, C30(6814/7212) MMTV DNA acts as an inducible, orientation-independent transcription-activating sequence when linked to the heterologous *tk* promoter. Further experiments with the same segments of clones C11 and 15.8 yielded a similar pattern of results, that is, orientation-independent transcriptional activation of the *tk* promoter (data not shown).

The origin of the CAT mRNA induced through activation of the META fragment was studied by RNase protection experiments. For reasons explained in Discussion, these experiments were carried out by using plasmid pBLCAT2-C30(7212-6814) as the source of the induced mRNA. This construct contains the META fragment in the reverse orientation, and the CAT mRNA would be expected to initiate from within the *tk* promoter. The radioactive probe RNA was synthesized from the vector pCM1 (Fig. 3). It was hybridized to mRNA from either EL4.E1 or Jurkat cells which had been transfected with pBLCAT2-C30(7212-6814) and was stimulated with ionomycin plus PMA. This combination was found to induce expression of the META constructs even more strongly than did ConA plus PMA, as has also been found for lymphokine gene transcription.

The results of the RNase protection experiments (Fig. 9) show that the origin of CAT mRNA is at or close to the transcriptional start site in the *tk* promoter. Induction for various times led to an increase of RNA which initiated at this site, in keeping with the results of the CAT assay itself.

DISCUSSION

We have identified a transcription-activating segment of proviral MMTV *env* genes, the META sequence, whose activity is inducible in T-helper cell lines but inactive in other types of cells. The activity of the META is sensitive to CsA in three of the four isolates characterized. These activating sequences thus mimic the transcription elements regulating expression of the IL-2 gene and certain other lymphokine genes. A number of the *env*-derived sequences from the T-lymphoma line EL4.E1, the T-helper hybridoma line 12.1.19, and BALB/c splenic DNA are inducible, but not all such sequences are active; some of the cloned 505-bp fragments from EL4.E1 were inactive in the transcriptional assay (data not shown). The basis for the differences presumably resides in sequence differences between various clones (Fig. 2) and is under investigation. The segment C30(6750/7255) likely contains multiple regulatory elements, including some which may be constitutively active in T cells. This appears to be the case for the *tk* promoter construct in pBLCAT2 (Fig. 8).

Nucleotide sequences for clones C30(6750/7255), C11(6750/7255), and 15-8(6750/7333) are compared with the sequence of the proviral form of the milk-borne virus (31) in Fig. 2. There are some 27 point differences between clones C11 and C30, which presumably account for the differences in their behavior (e.g., the greater suppressibility of C30 by CsA). There is a TATA box located some 30 bases upstream of the start site of the META-regulated transcript and a CCAAT sequence at about −50. There are two elements which are similar to regulatory elements of the IL-2 gene and which may account, in part, for the induction of the META transcript. These are the TCEp segment (41), centered at 6980 (−266 relative to the start of transcription), and the purine box (43) at 7040 (−206). The TCEp element in the IL-2 gene (at −172 in the mouse gene) shares binding with AP-3 elements, but upon induction of EL4 cells with PMA, a separate factor (other than AP-3) binds to it (41). The precise boundary of this element is not defined, but it probably ends as indicated in Fig. 2, since immediately following this there is a different element, which binds a factor induced by stimulation through the CD28 surface antigen (16) and which is not present in the MMTV sequences. The block of purines at 7040 is similar to the conserved purine block found in the mouse and human IL-2 genes and in a number of other cytokine genes (18). In the IL-2 gene, the purine block is within a larger segment (not present in MMTV) defining the critical NF-AT site, which is of primary importance in regulating IL-2 gene transcription (43).

Although the META segment contains a transcriptional start site in EL4.E1 cells (Fig. 4) (13), we have not established whether the transcripts encoded by all of the CAT constructs begin at the same position. We encountered a fundamental difficulty in making such a determination. Namely, the level of CAT mRNA molecules was very low in transfected cells, and primer extension experiments were not sensitive enough to detect their starting points (data not shown). RNase protection is more sensitive, but in EL4.E1 cells, we found that probes which contained MMTV antisense sequences hybridized to many endogenous RNAs and generated a high background of protected probe fragments. This finding is not surprising, since there is constitutive expression of various MMTV RNAs. RNase protection with a sense-strand probe did not show such a background, also as expected, but could not be used to detect CAT mRNA initiation sites which are within the META fragment itself, since for these sites to work, they must be in the forward orientation. However, in the case of the 400-bp META fragment, which functioned as an activator of the *tk* promoter in the reverse orientation [i.e., C30(7212-6814)], we were able to detect an appropriate start site for the corresponding CAT mRNA (Fig. 9).

The META fragment cloned from 12.1.19 cells (clone C11) was active in the transfection assay in all of the activated T-helper cells studied, including the original 12.1.19 cells themselves (Fig. 6 and 7). However, we have been unable to detect endogenous activity of the META promoter in these cells under any circumstances. This lack of activity may be due to down-regulation by elements within the LTR in the endogenous genome. There are several reports indicating that the MMTV LTR does, in fact, contain negative control elements for transcription from the conventional, glucocorticoid-induced LTR promoter, which is located near the 3′ terminus of the LTR. For example, removal of a 91-bp fragment immediately upstream of the glucocorticoid response elements in a CAT construct enhanced the LTR promoter's activity upon hormone induction (33). Second, the EL4 LTR, which has a deletion of 494 bp relative to the milk-borne virus sequence, drove expression of the LTR promoter in transgenic mice in tissues other than those active in constructs carrying the complete LTR (38). Furthermore, when linked to a CAT gene and transfected into cells, the EL4 LTR produced a sevenfold-higher level of expression than the intact LTR did (23). LTR-deletion variants of MMTV were also found to be inducible by PMA

in T-cell lines, whereas the normal LTR sequences were not (45). There is also evidence that the LTR ORF encodes a negative-acting factor which partially inhibits transcription from a heterologous (RSV) promoter (39). These and other data (35) strongly indicate that the LTR of MMTV contains a negative transcriptional element(s). Whether it also affects the META promoter is not yet known. Of course, all of the constructs studied in this work are completely dissociated from any *cis* effects of the putative LTR silencer.

It is striking that deletions in the same central region of the MMTV LTR are characteristic of many T lymphomas (30, 49), of certain other MMTV-related tumors (47), and of a thymoma-inducing variant of MMTV itself (3). If the removal or inactivation of this region of the LTR disinhibits nearby promoters generally, this might account for the endogenous META activity seen in EL4.E1 cells but not in 12.1.19 cells. This effect may also be related to the frequency of LTR deletions in T lymphomas and to the T-cell tropism of the MMTV viral variant DMBA-LV (2, 3), which lacks the central part of the LTR. In a different system, murine leukemia virus, a switch in tropism to T cells was found to be caused by alterations in the U3 region (6).

The product of META-driven transcription in the MMTV proviral genome is the LTR region itself, which contains a long ORF. Recent work indicates that this ORF encodes the gene product which determines the Mls locus in mice (15, 29, 36). Our data suggest a basis for lymphocyte-specific expression of this region, that is, a promoter-enhancer which responds to activation signals in T lymphocytes and which transcribes the putative Mls locus. Although expression of Mls as antigen is generally highest on B lymphocytes and other antigen-presenting cells, T cells are far more effective in presenting this superantigen in a tolerogenic context (46). $CD4^+$ (T-helper) T cells are less effective than $CD8^+$ (cytotoxic-suppressor) T cells, although either will suffice in vivo. Induction of self-tolerance to the Mls system could thus be dependent on T-cell-specific expression of the LTR of MMTV, for which the META site would be a logical activator, since it directs the transcription of the Mls-determining ORF. Treatment of neonatal mice with CsA prevents apoptosis in the thymus (44) and allows the emergence of self-reactive clones, including ones expressing the I-E-reactive Vβ11-T cell receptor (19). Presentation of this self antigen requires an essential coligand, identified as Etc-1, which has been found to be the superantigen encoded by the MMTV LTR (48). The lack of self-tolerance for I-E induced by CsA could thus be explained by an effect similar to that described here: inhibition of META-driven transcription of the MMTV LTR (i.e., Mls), which leads to a loss of anti-self-tolerance in the thymus.

The LTR ORF appears to encode a transmembrane glycoprotein, which traverses the plasma membrane at a hydrophobic site between amino acids 45 to 63 (8). The site of Mls antigenicity is probably within the carboxyl-terminal variable block between residues 290 and 320 (8, 36). If the deleted variants of MMTV found in T lymphomas were expressed similarly, they would present a shortened extracellular domain carrying about 13,000 Da of conventional ORF sequence but lacking the Mls antigenicity. The effects of a high level of such expression are not known, although this protein has been detected in stimulated EL4 cells (37).

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