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Relationship between an enhancer element in the human antithrombin III gene and an immunoglobulin light-chain gene enhancer

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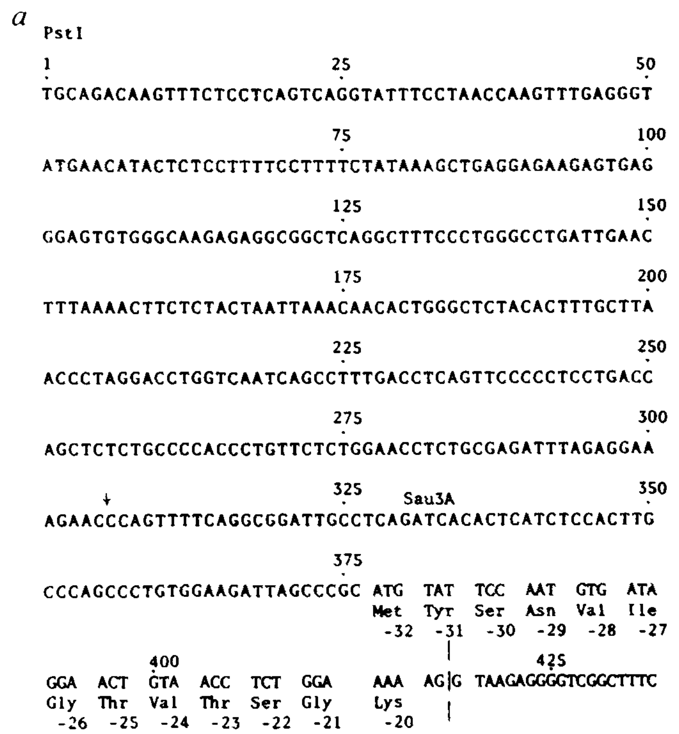
Enhancers, *cis*-acting transcriptional control elements have been described in both viral and cellular genes[1]. They influence transcription in a quantitative fashion, act over relatively large distances (several kilobases, kb) and behave independently of their position and orientation. Enhancers have been described in immunoglobulin, chymotrypsin and insulin genes[2-9]. They bear little homology with each other except for an 8-base pair (bp) 'consensus' core element, GTGG$^{AAA}_{TTT}$G (refs 10, 11), but even this element is sometimes non-homologous[9]. I have searched for such elements in the human antithrombin III (AT-III) gene. AT-III is an important coagulation protein which inactivates thrombin[12]. It is produced by the liver and, to a lesser extent, by the kidney[13]. Here, I report that the 5' flanking region of the AT-III gene encodes a segment homolgous with the enhancer containing the joining–constant$_\kappa$ (J_κ–C_κ) intron of immunoglobulin κ-chain genes. This extensive homology suggests the existence of regulatory factors that recognize common DNA sequences in lymphoid tissues and in those which express AT-III.

Two regions in the 5'-flanking region of the AT-III gene demonstrate sequence homology with sites in the J_κ-C_κ introns of murine or human immunoglobulin C_κ genes (Fig. 1). Region 1 corresponds to nucleotides 252-264 of the AT-III gene[14] and to nucleotides 3,904-3,916 of the J_κ-C_κ intron of human and murine C genes[15,16]. Ten of 13 residues (77%) were homologous in the AT-III and human J_κ-C_κ sequences and 9 of 13 residues (69%) were homologous in the AT-III and murine J_κ-C_κ sequence. A second, more extensive, region of homology (17 of 22 bases, 77%) was observed between nucleotides 291 and 312



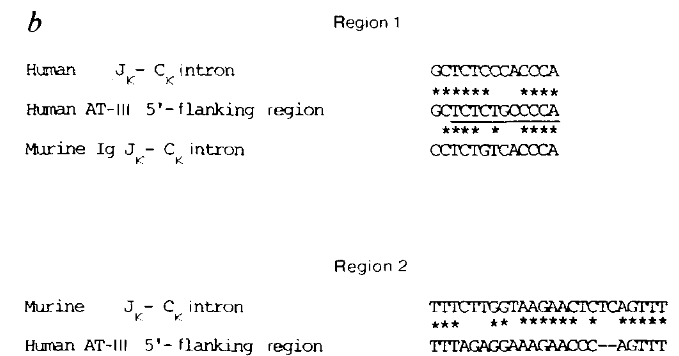


Fig. 1 Homologies between the human AT-III 5'-flanking region and human and mouse J_κ-C_κ large introns. *a*, DNA sequence of the immediate 5' end of the AT-III gene. The sequence shown here is a portion of a 1.2-kb *Pst*I genomic subclone (pAT1.2) containing 304 bp upstream flanking region, 72 bp 5'-untranslated region and codons for the first 13 amino acids of the signal peptide[14]. The rest of the subclone contains ~800 bp of intervening sequence. The arrow at position 304 denotes the transcriptional start site. *b*, Sequence homologies between AT-III and J_κ-C_κ introns. In region 1, the AT-III sequence shown is that between nucleotides 252 and 264. The J_κ-C_κ sequence is that between nucleotides 3,904 and 3,916, according to the numbering system of Max *et al.*[16]. In region 2, the AT-III sequence is that between nucleotides 291 and 312 and the J_κ-C_κ sequence is that between nucleotides 3,868 and 3,892 (refs 15, 16). Underlined regions correspond to polyoma virus or core enhancer sequence homologies[17-19].

of AT-III and nucleotides 3,869-3,892 of the murine J_κ-C_κ region, although homology with the human J_κ-C_κ region was not observed. Although regions 1 and 2 are short and homology could conceivably be fortuitous, it was striking that both regions bear similarities to known viral enhancer elements. For example, in region 1, the underlined area (Fig. 1*b*) is homologous with a portion of the polyoma virus enhancer TCTCCACCA[17-19]. Similarly, the underlined area in region 2 is homologous with the 'core' consensus sequence GTGG$^{AAA}_{TTT}$G, thought to be[10,11] common to some eukaryotic viral enhancer elements. Thus, the 5'-flanking region of the human AT-III gene might contain functional enhancer activity; I tested this suggestion in an *in vivo* transfection assay using the chloramphenicol

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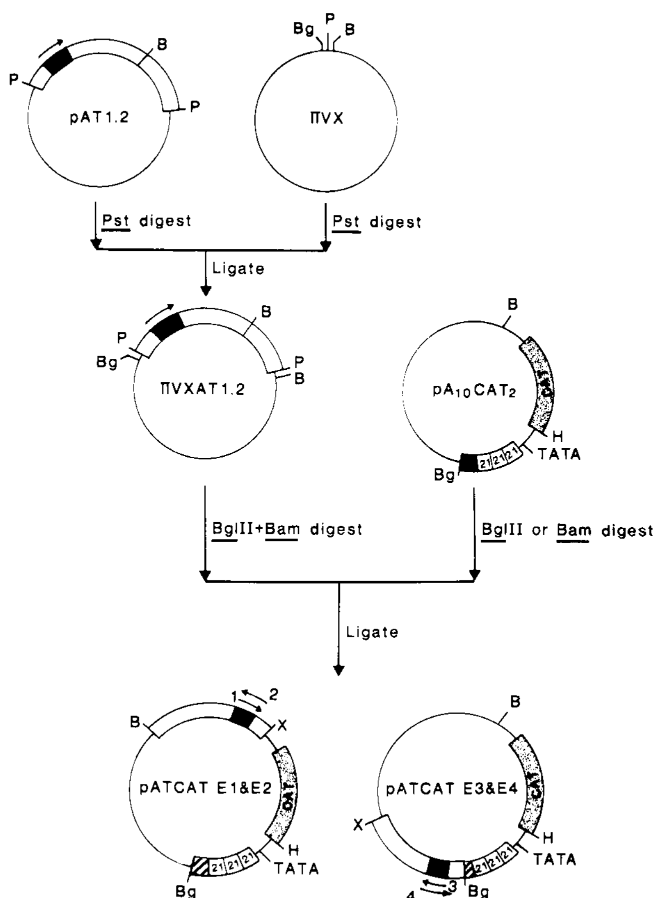

Fig. 2 Construction of AT-III CAT vectors. pAT1.2 is the 1.2-kb AT-III genomic sublcone described in Fig. 1 legend. The insert was excised with *Pst*I and ligated into the miniplasmid πvx[37] in such an orientation that the region containing the enhancer element was bounded by a 5′ *Bgl*II site in the πvx polylinker and the 3′ *Bam*HI site in IVS-1 of the AT-III gene. Following digestion of πvxAT1.2 with *Bgl*II and *Bam*HI, the 800-bp-long enhancer-containing fragment was ligated in both possible oreintations into the *Bgl*II (upstream) or *Bam*HI (downstream) sites of the enhance-less CAT expression vector $pA_{10}CAT_2$. The resulting four constructs were designated pATCAT-E1-E4, with only orientations 1 and 3 shown here for simplicity. The arrows indicate the 5′→3′ direction of the enhancer element in each of the constructs. Note that the relative proximities of the enhancer element to the CAT promoter are therefore E3 > E4 > E2 > E1. P, *Pst*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; X, *Bam*HI or *Bgl*II sites destroyed by ligation of nonregenerative GATC sticky ends. Before transfection studies, all plasmids were isolated by the alkaline lysis method[38] and doubly banded in CsCl to ensure complete removal of contaminating nucleic acids. Open box, AT-III-derived sequences; solid box, J_κ-C_κ enhancer -like region; solid lines, plasmid-derived sequences; stippled box, CAT gene; cross-hatched box; truncated SV40 enhancer region; 21, SV40 21-bp repeats.

acetyltransferase (CAT) assay[20]. Restriction fragments containing the putative enhancer element from the AT-III gene were cloned into the enhancerless vector $PA_{10}CAT_2$ (Fig. 2) and their effect on transcription quantitated by examining cell extracts for the presence of CAT following transfection. A positive control plasmid, pSV_2CAT, contains a simian virus 40 (SV40) enhancer and directs the synthesis of large amounts of CAT. Each plasmid was transfected into four different eukaryotic cell lines, two of which, the COS-1 African green monkey kidney line[21] and the Alexander human hepatoma line[22] are derived from tissues that normally express AT-III (ref. 13). Cell extracts were prepared and assayed for CAT activity 48 h later[20]. CAT activities were normalized to the results of Hirt extracts[23] to allow for variations in the transfected DNA uptake and replication. The results (Fig. 3) can be summarized as follows: (1) CAT activity was significantly enhanced above background by the presence of 800 bp of AT-III gene sequence containing the J_κ-C_κ homologies; (2) the level of enhancement was generally in inverse proportion to the distance of the putative enhancer element from the SV40 early promoter, although some variability was seen between experiments; (3) enhancer activity was independent of the orientation of the AT-III sequences; (4) enhancer activity was seen only in those cells derived from tissues that naturally produce AT-III, that is, COS-1 (kidney) and Alexander hepatoma (liver). Examination of Hirt extracts of transfected cells showed only small differences in plasmid copy number within any single experiment, indicating that the increased CAT activity was not the result of preferential replication of ehancer-containing constructs in COS-1 cells or of variability in DNA uptake (my unpublished data). Therefore, I conclude that the 5′ end of the human AT-III gene contains sequences that potentiate hereologous gene transcription in a tissue-specific manner when assayed by transient expression.

The extensive sequence homology observed between AT-III and immunoglobulin J_κ-C_κ enhancers suggested that there is also functional homology. This possibility was tested by introducing a murine immunoglobulin J_κ-C_κ enhancer into Alexander and COS-1 cells and the AT-III enhancer into a murine myeloma line and measuring CAT activity 48 h later. In the former case, the immunoglobulin J_κ-C_κ enhancer element was cloned into the *Bgl*II site of $pA_{10}CAT_2$. The resultant plasmid, designated $pA_{10}CAT_2$-κ, was then introduced into Alexander or COS-1 cells by calcium phosphate precipitation. The plasmids $pA_{10}CAT_2$-κ and pATCAT-E3 were transfected into 10^7 M12 murine myeloma cells using the electroporation technique[24] and cell extracts were assayed for CAT after 48 h (Table 1). As observed previously, the AT-III enhancer functioned in both Alexander and COS-1 cells. In a similar fashion, $pA_{10}CAT_2$-κ also functioned above background when introduced into M12 cells. More significant, however, was the observation that the AT-III enhancer functioned in M12 cells and that the immunoglobulin J_κ-C_κ enhancer functioned in at least one of the cell lines (COS-1) derived from AT-III-producing tissues. CAT activity was generally less in the heterologous cells: the AT-III enhancer functioned better in COS-1 and Alexander cells whereas the J_κ-C_κ enhancer functioned better in M12 cells. $pA_{10}CAT_2$-κ functioned at only

Table 1 Relative activity of AT-III and J_κ-C_κ enhancers in myeloma cells, hepatocytes and COS-1 cells

Transfected plasmid	M12 myeloma	CAT activity in Alexander hepatoma	COS-1
pATCAT-E3	4.0	4.3	12.8
$pA_{10}CAT_2$	7.2	1.5	4.4
$pA_{10}CAT_2$	1.0	1.0	1.0

Plasmid pATCAT-E3 is described in Fig. 2 legend. $pA_{10}CAT_2$-κ was constructed by removal of the 1.25-kb *Hin*dIII/*Hpa*I fragment from the rearranged murine gene MOPC-41κ[16]. The fragment was repaired with DNA polymerase (Klenow fragment) and deoxynucleoside triphosphates. *Bgl*II linkers were added, followed by ligation into the *Bgl*II site of $pA_{10}CAT_2$. The orientation was such that the former *Hin*dIII site was brought closer to the SV40 early promoter. 20 μg of each plasmid was introduced into either Alexander or COS-1 cells by standard calcium phosphate DNA co-precipitation onto 5×10^5 cells plates the day before. For M12 cells, transfection was accomplished by electroporation of 10^7 myeloma cells in 0.5 ml phosphate-buffered saline (PBS). The procedure was essentially as described previously[24] using an Isco Model 494 electrophoresis power supply. Voltage was adjusted to 2,000 V, current to 0.9 mA and the adjustable current and wattage set at a minimum. Cells were kept at 0 °C in PBS during electroporation and for 10 min afterwards before being replated in modified Eagle's medium (NEM)/10% fetal calf serum (FCS). Following transfection, each cell line was grown for 48 h in MEM/FCS, at which time CAT activity was measured in extracts. The values shown represent the increase over the amount of CAT conversion seen after transfection with $pA_{10}CAT_2$ alone (value arbitrarily set at 1).

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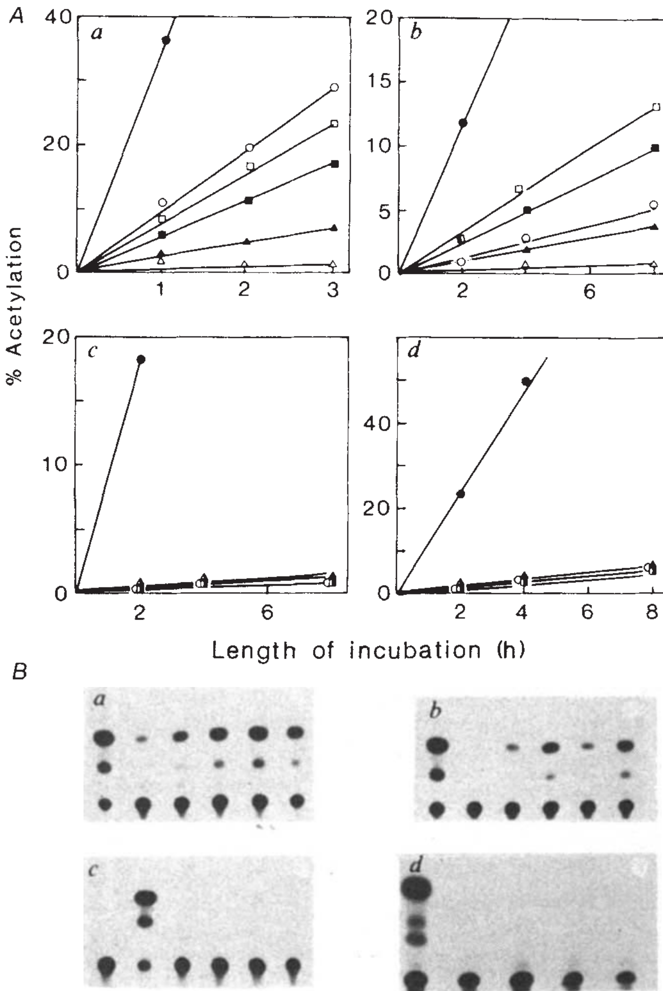

Fig. 3 Tissue-specific CAT expression. *A*, Kinetics of CAT activity in four different cell lines: *a*, COS-1; *b*, Alexander human hepatoma; *c*, HeLa; *d*, mouse thymidine kinase-negative L cells. Transfections were with pSV_2CAT (●); $pA_{10}CAT_2$ (△); pATCAT-E1 (▲); pATCAT-E2 (■); pATCAT-E3 (○); pATCAT-E4 (□). All points in this and subsequent experiments are normalized for differences in plasmid copy number as determined by DNA dot-blots of Hirt extracts[23]. *B*, Thin-layer chromatograms of CAT conversion products. Spots nearest the bottom represent unacetylated ^{14}C-chloramphenocol. *a*, COS-1; *b*, Alexander; *c*, HeLa; *d*, L cells. From left to right in each lane, the products from transfections with pSV_2CAT, $pA_{10}CAT_2$, pATCAT-E1, pATCAT-E2, pATCAT-E3 and pATCAT-E4, respectively, are spotted. In *c* the $pAT_{10}CAT_2$ and pSV_2CAT lanes have been reversed. Each set of experiments was performed at least twice with three different plasmid preparations.

Methods. All cells were grown in Dulbecco's modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 $\mu g\ ml^{-1}$ streptomycin and 100 U ml^{-1} penicillin G. Cells were split into 100-mM tissue culture plates at 5×10^5 cells per plate and 24–48 h later, 20 μg of each of the four AT-III CAT constructs shown in Fig. 2 were introduced into the cells by calcium phosphate precipitation[39]. As negative and positive controls, $pA_{10}CAT_2$ or the SV40 enhancer-containing plasmid pSV_2CAT were also used[20]. Precipitates were kept on the cells for 5 h, after which the cells were washed with MEM and subjected to a 25% glycerol/MEM shock for 1 min; 48 h later, cells were washed in Tris-buffered saline, and scraped from each plate. An aliquot was used for Hirt extracts[23]. Hirt supernatants were dot-blotted onto nitrocellulose[40] and hybridized with a nick-translated pAT1.2 insert (specific activity 3×10^8 d.p.m. μg^{-1}). Hybridizations were carried out overnight at 68 °C in a solution consisting of 6×SSC, 5× Denhardt's solution, 1% SDS, 10 mM phosphate buffer *p*H 7.0, 1 mM EDTA, 100 $\mu g\ ml^{-1}$ sonicated salmon sperm DNA, 10% dextran sulphate (Pharmacia) and 10^6 d.p.m. ml^{-1} of nick-translated probe. The remaining cells were subjected to one cycle of freeze-thawing and then sonicated in 100 μl 250 mM Tris-HCl, *p*H 7.8. In the case of COS-1 cells, 20 μl aliquots of clarified extract were assayed for CAT activity, whereas in all other cases 50 μl was used. The assay system was that described by Gorman *et al.*[20], which relies on the conversion of ^{14}C-chloramphenicol to its acetylated derivatives and their resolution by silica gel TLC.

modest levels above background when introduced into Alexander cells. These experiments suggested that two unrelated genes can be expressed in the same tissues if they share homology in enhancer regions.

It was possible that the effects seen with the AT-III or immunoglobulin gene fragments were the result of the introduction of cryptic promoter elements into the $pA_{10}CAT_2$ vector. To address this issue, I performed a series of primer extension experiments. An end-labelled CAT-specific *Eco*RI/*Pvu*II restriction fragment was strand-separated and hybridized with toal RNA from COS-I cells that had been transfected with CAT plasmids 48 h previously. The DNA/RNA hybrid was then extended with reverse transcriptase and the size of the product analysed by polyacrylamide gel electrophoresis (Fig. 4). Transcripts initiating at the SV40 early promoter should have extended products 307 bp in length, whereas those originating from upstream sequences should be longer. Transcripts initiated correctly in each of the plasmids tested regardless of whether they contained SV40, AT-III or immunoglobulin upstream sequences.

An understanding of the developmental and/or tissue-specific control of eukaryotic gene expression will ultimately require an appreciation of the various factors whose interactions influence transcription. Specific DNA sequences are known to affect gene transcription, presumably by serving in a manner analogous to classical prokaryotic promoters in allowing for binding of RNA polymerases[25-29] The more recent descriptions of enhancers have defined a new class of transcriptional control signals, often with tissue-specific regulatory capacity. That enhancers function irrespective of position and orientation[1,27-29] has given rise to the notion that, by disrupting chromatin structure, they serve as preferred entry points for RNA polymerases which then scan adjacent DNA sequences until encountering a promoter element[30-32]. Here, I provide evidence for an enhancer element at the 5′ end of the human AT-III gene. Its presence was initially inferred on the basis of homologies between the AT-III gene and the J_κ–C_κ introns of murine and human immunoglobulin genes which contain tissue-specific enhancers. In addition to their homologies to viral sequences, however, the AT-III and immunoglobulin J_κ–C_κ genes share more extensive homologies with one another. That these homologies occur in close physical association with the polyoma virus-like and viral core sequences suggests that they are involved in the tissue-specific regulation of AT-III expression.

Immunoglobulin genes function poorly, if at all, in nonlymphoid cells such as fibroblasts[3,4]. However, because of the close structural homology between AT-III and J_κ–C_κ enhancers, I tested for functional homology by introducing a murine J_κ–C_κ enhancer into Alexander hepatoma cells and the AT-III enhancer into myeloma cells. In both cases increased CAT activity was seen although the magnitude of the enhancement was low. This may reflect a suboptimal interaction between the enhancers and tissue-specific factors[33] or the need for additional factors that are absent from the heterologous cells. The J_κ–C_κ enhancer showed low activity even in myeloma cells and is consistent with the findings of Picard and Schaffner[5] who demonstrated that the murine κ-chain gene enhancer is only 5% as effective as heavy-chain gene enhancers in promoting heterolgous gene transcription.

Extensive homologies among enhancers have not previously been reported. Laimins *et al.*[10] and Weiher *et al.*[11] have noted a consensus core sequence (GTGG$^{AAA}_{TTT}$G) in several viral enhancer elements but additional sequence homology was not observed. Walker *et al.*[9] reported that the sequence GTGGAAA was found in fragments with enhancer activity from the 5′-flanking region of human and rat insulin genes although the

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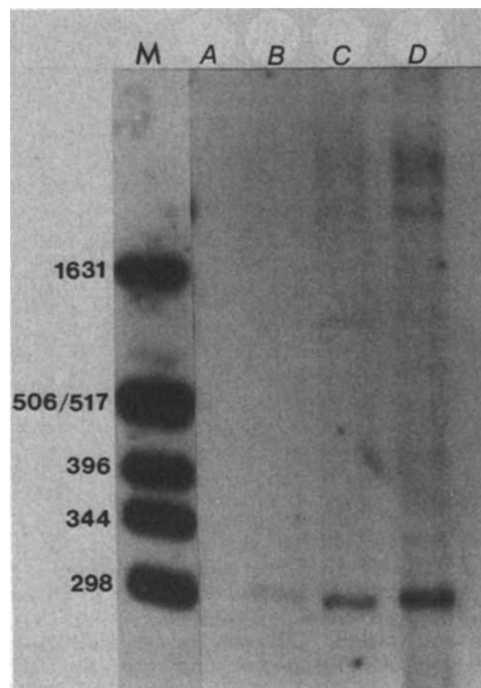

Fig. 4 A primer extension analysis of CAT transcripts in COS-1 cells. M, Marker. Primer extended products from cells transfected with: *A*, $pA_{10}CAT_2$; *B*, $pA_{10}CAT_{2-\kappa}$; *C*, pATCAT-E3; *D*, pSV_2CAT. **Methods.** Transfections were performed with 20 μg per plate of each plasmid tested ($pA_{10}CAT_2$, pSV_2CAT, pATCAT-E3 and $pA_{10}CAT_2$-K). Cells were collected after 48 h and total RNA was extracted by the guanidine hydrochloride method[39]. 25 μg RNA was hybridized with 80,000 d.p.m. of a 104-nucleotide-long *Eco*RI/*Pvu*II strand-separated restriction fragment from the CAT gene which had been labelled at the *Eco*RI site to specific activity of 2×10^7 d.p.m. μg^{-1} using polynucleotide kinase and [γ-^{32}P]ATP (specific activity 7,500 Ci $mmol^{-1}$; Amersham). Hybridization conditions were: 80% formamide, 280 mM NaCl, 60 mM PIPES, *p*H 6.8 in 10-μl reactions at 51 °C for 16 h. Hybrids were then phenol-extracted, precipitated and redissolved in 25 μl 70 mM KCl, 10 mM Tris, *p*H 8.2, 8 mM $MgCl_2$, 10 mM dithiothreitol, 0.25 mM of each deoxynucleoside triphosphate and 50 U avian myeloblastosis virus reverse transcriptase. After 60 min at 42 °C, the reactions were phenol-extracted, precipitated, redissolved in formamide dye buffer and electrophoresed through an 8% polyacrylamide/8 M urea sequencing gel.

sequence was not required for optimal activity. Therefore, the homology between AT-III and J_κ-C_κ enhancers is striking in view of the minimal relatedness of other enhancers.

In an effort to correlate more precisely the J_κ-C_κ homology with enhancer activity, I have tested a series of deletion plasmids, one of which contains only 177 bp of AT-III sequence, including the immunoglobulin enhancer homology (nucleotide 153-330; Fig. 1). When tested in COS-1 cells, this construct is as active as any of the constructs shown in Fig. 2 (unpublished). Current experiments are focused on the construction of additional deletions to determine whether the same or different sequences are required for full expression in COS and Alexander cells.

The significance of the homologies reported here is not yet clear. It may be that cellular genes contain only a limited number of enhancer elements many of which have fairly extensive homology with each other despite widely different functions of the genes they control. AT-III and light-chain genes might fortuitously represent members of one such subset of enhancers. Alternatively, as B cells are thought to originate in the liver during early embryogenesis[34,35], these enhancers might represent common tissue- or stage-specific targets for cellular transcription factors. A distinction between these and other alternatives should be possible when more cellular enhancers have been characterized. In either case, it seems unlikely that the homologies noted here represent the entire region responsible for tissue specificity. Despite the fact that the AT-III and J_κ-C_κ enhancer elements can function in heterologous cells, it is clear at least for immunoglobulin genes that this is not normally the case[36]. Other unique cellular factors and/or DNA sequences are likely to be responsible for conferring additional tissue specificity on gene expression.

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Local cytoplasmic calcium gradients in living mitotic cells

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Cytoplasmic free calcium has been proposed as a regulator of many microtubule-mediated processes, including mitosis. It has been difficult to test this hypothesis because methods for local measurement of free Ca^{2+} in the living cell have not been available. We have used the fluorescent calcium indicator dye Quin-2 (methoxyquinoline - 1bis(*o* - aminophenoxy)ethane - *N*,*N*,*N′*,*N′* - tetra acetic acid), which allows such observations to be made by digital processing of fluorescent images from the light microscope. Here

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