

species found in these cells. The complete characterization of c-sis cDNA clones, as well as experiments designed to elucidate the precursor-product relations of the various proteins, should help to determine the relevance of these proteins to the synthesis, assembly, and function of PDGF in U-2 OS cells.

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16. Antiserum to PDGF was produced in rabbits as described by A. J. Owen *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **79**, 3203 (1982)]. Confluent U-2 OS cells were incubated for 2 hours in cysteine-free MEM containing 300 μ Ci of carrier-free [35 S]cysteine, per milliliter. The cells were collected ($\sim 10^7$ per batch) and lysed with immunoprecipitation buffer [0.3M NaCl, 0.02M tris-HCl (pH 7.5), 0.001M EDTA, 0.01M NaI, 0.002M phenylmethylsulfonyl fluoride (PMSF), 0.3 percent Triton X-100, and 1 percent human serum albumin (HSA)]. The extracts were cleared with 50 μ g of Protein A-Sepharose. Normal rabbit serum or antiserum to PDGF (2 μ l) was then added to equal portions of the cell extract and allowed to incubate for 18 hours at 4°C. Immune complexes were recovered by the addition of Protein A-Sepharose (40 μ g).
17. The washed Protein A-Sepharose beads were resuspended in SDS sample buffer [50 mM tris-HCl (pH 6.8), 2 percent SDS, 1 mM PMSF, and 12 percent glycerol] and boiled for 3 minutes. The beads were pelleted, and the supernatants were collected and divided into two equal portions. Dithiothreitol (100 mM final concentration) was added to one of the samples and left for 90 minutes at 37°C. 2-Mercaptoethanol (10 percent final volume) was added to the reduced sample 15 minutes before applying the sample to gels (30 by 20 cm by 0.8 mm SDS-PAGE). Resolving gels (8 percent) were used to analyze the large peptides and 16 percent gels for the small peptides. The gels were run as described [P. Pantazis and W. M. Bonner, *J. Biol. Chem.* **256**, 4669 (1981)]. Gels were dried and autoradiographed as described [W. M. Bonner, M. H. P. West, J. D. Stedman, *Eur. J. Biochem.* **109**, 17 (1980)].
18. Labeled conditioned medium from U-2 OS cells was prepared and immunoprecipitated as described by A. J. Owen *et al.* [*in (12)*].
19. CM-Sephadex that had been swollen was added to MEM conditioned with U-2 OS cells (100 ml), dialyzed overnight against water, poured on a column, and eluted with 1M NaCl. The eluted fractions were dialyzed exhaustively against acetic acid, lyophilized, and resuspended in HSA (1 percent).
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18 May 1984; accepted 27 July 1984

Functional Insertion of an Alu Type 2 (B2 SINE) Repetitive Sequence in Murine Class I Genes

Abstract. The regulation of expression of the family of MHC (major histocompatibility complex) class I genes is complex. Sequence analysis has revealed that class I genes from the H-2D subregion of the MHC (which includes the D and L genes) differ from the class I gene from the H-2K subregion (the K gene) by the insertion of a type 2 Alu-like repetitive element (the murine B2 sequence) within the 3' noncoding region of the D and L genes. The consequence of this insertion in the D and L genes is the introduction of a novel polyadenylation signal, which is preferentially used over the more distal signal, the analog of that found in the K gene. The insertion of the type 2 Alu-like sequence results in a change in the preferred site for endonucleolytic cleavage which is necessary for generating a correct 3' terminus for polyadenylation. The data demonstrate that the type 2 Alu-like sequence has a function; the data also suggest a possible regulatory role of this sequence in the expression of class I genes.

About 25 to 35 class I genes have been mapped to the major histocompatibility complex (MHC) of different mouse strains (1). Those class I genes located on the centromeric side of the MHC encode polymorphic cell-surface trans-

plantation antigens (designated K, D, and L) which play a critical role in inducing graft rejection and in permitting cytotoxic T cells to distinguish self from nonself (2). Other class I genes located in the Qa2,3 and Tla regions on the telomeric side of the MHC encode structurally related antigens that differ in their tissue distribution and possibly in their function (3).

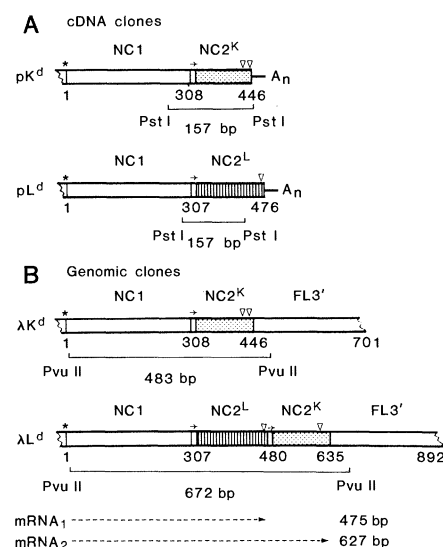


Fig. 1. Diagram of the organization at the 3' ends of class I cDNA and genomic clones. The cDNA structures (designated p) for K^d and L^d were derived from pH2^d-4 and pH2^d-3⁽⁵⁾, respectively. The genomic structures (designated λ) for K^d and L^d were from λ2.14 (23) and λL^d-D5. The noncoding region 1 is designated NC1, the noncoding region 2 as NC2, the poly(A) (polyadenylated) tract as An, and the 3' flanking sequences as FL3'. Analogous but nonhomologous regions are indicated by stipples and stripes. The asterisks denote the TGA translational termination codons, the arrows indicate the direct repeats, and the triangles identify the polyadenylation signals. Sequences are numbered with reference to the nucleotide immediately following the TGA termination codon.

All the class I genes are homologous in sequence. The conservation in amino acid sequence observed in different gene products is about 80 percent. However, analysis of complementary DNA (cDNA) sequences has suggested that different class I transcripts can be distinguished by the presence of unique sequences at their 3' ends (4, 5). Within the 3' noncoding regions of all class I transcripts thus far analyzed, approximately the first 300 nucleotides (designated noncoding region 1 or NC1) seem to be

highly conserved, whereas the following ~140 to 170 nucleotides (designated noncoding region 2 or NC2) appear to be completely divergent (6). For example, the NC1 regions of the K and L genes are about 87.5 percent homologous in sequence, yet their NC2 regions share no detectable homology (see Fig. 1A). Similarly, the class I-related transcript, which encodes a secreted antigen, has a highly conserved NC1 sequence but an NC2 sequence, which is only distantly related to that of the K gene (7). The

same is true for the 27.1 pseudogene that was derived from the Qa region of the MHC (8). The only exception to a divergence in the NC2 region of the various class I genes is that observed between the D and L genes, whose NC2 regions are virtually identical in sequence (6). Since the L gene is not present in all mouse strains, and when present is always closely linked to the D gene within the H-2D subregion of the MHC, it has been suggested that the L gene may have arisen by a recent duplication of the D

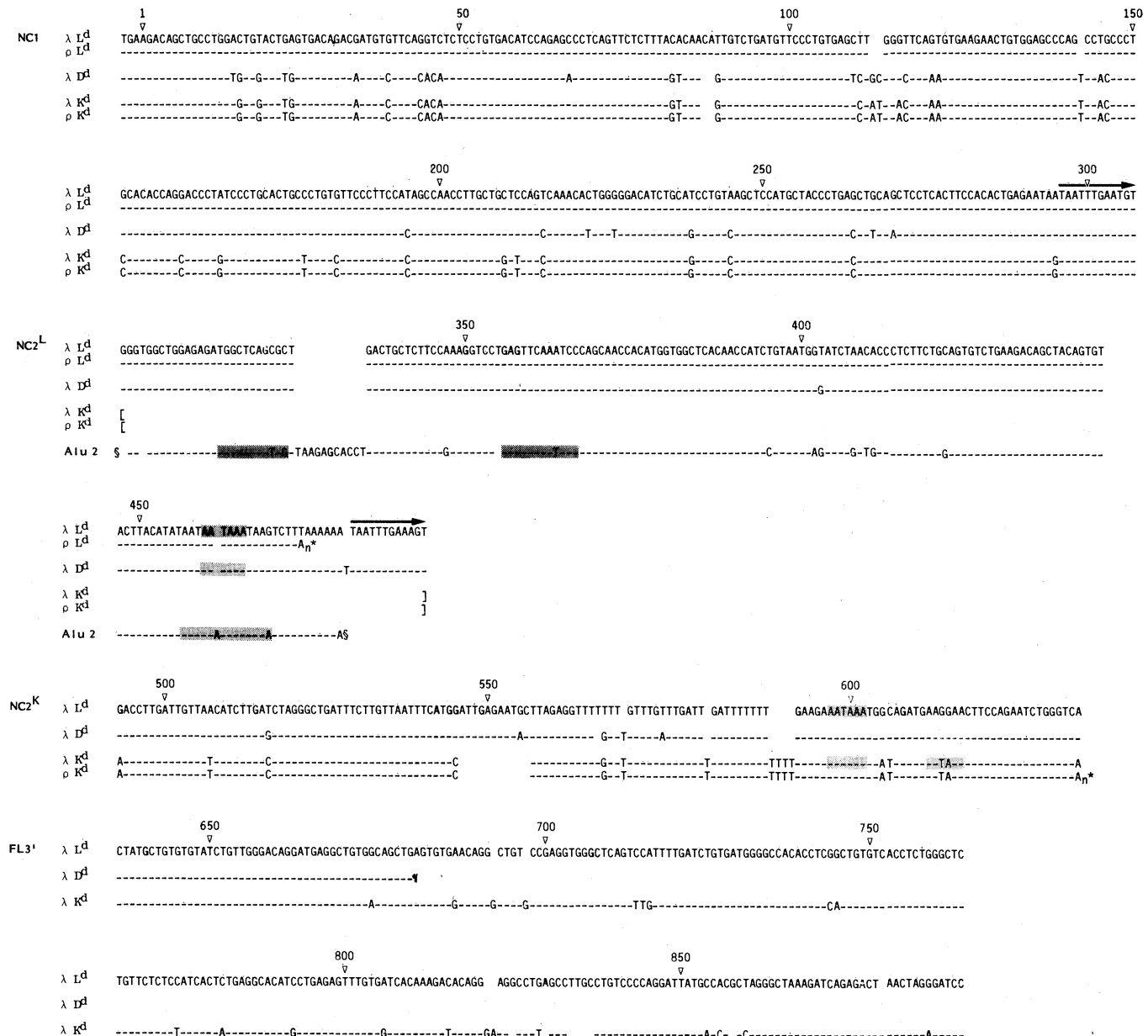


Fig. 2. Comparison of sequences distal to the translational termination codons of different class I genes. The genomic sequences (designated λ) of L^d (λL^d -D5) and D^d (λD^d -4) were determined by the primer-extension method in the presence of 2',3'-dideoxynucleoside triphosphates (24). The K^d sequence was from $\lambda 2.14$ (23). In this comparison, the λL^d sequence was chosen as the prototype with which λD^d and λK^d were compared. Also included are the cDNA sequences (designated p) from the L^d (pH2^d-3) and K^d (pH2^d-4) genes (5). Only positions where a substitution has occurred are indicated. The numbering is with reference to the nucleotide that immediately follows the TGA termination codon. The arrows show the location of the direct repeats, and the asterisks denote the end of the cDNA sequences. Also indicated are the locations of the RNA polymerase III split promoters (dark shading) and the polyadenylation signals (light shading). The D^d sequence has not been completed and stops at the Pvu II site as indicated. The entire sequence is contiguous but is presented as four consecutive segments designated noncoding region 1 (NC1), noncoding region 2 (NC2^L), noncoding region 2 (NC2^K), and 3' flanking region (FL3'). The consensus type 2 Alu-like sequence (19) is included with the NC2^L sequences for comparison.

gene. Thus, the two genes are not expected to be distinguishable by their NC2 sequences.

Unique sequence probes derived from the NC2 regions of different class I cDNA clones by digestion with Pst I have been successfully used for the analysis of specific class I transcripts (9). However, analysis of genomic clones isolated from a bacteria phage lambda library derived from BALB/c (H-2^d haplotype) embryonic DNA with these probes revealed an unexpected result. The NC2^K probe identified not only the K^d gene (λ K1) but also the L^d gene (λ D5). The assignment of these two cloned genes were both by the use of locus-specific synthetic oligonucleotide probes (6) and by analysis of the expression of K^d and L^d specificities with the use of monoclonal antibodies on mouse L cells (H-2^k haplotype) transfected by these genes (1). Although digestion of clone λ K1 with Pvu II yielded a ~480-bp fragment that hybridized to only NC2^K but not to NC2^L, digestion of clone λ D5 with the same restriction enzyme gave a ~670-bp fragment that hybridized to both NC2^K and NC2^L. This observation is totally unexpected on the basis of the analysis of cDNA sequences (Fig. 1A).

In order to determine the precise arrangement of the NC2^K and NC2^L nucleotides, we sequenced the segment of the L^d gene that spanned the 3' noncoding region and its neighboring 3' flanking region and compared it to the corresponding segment of the K^d gene (Fig. 2). The deduced sequences reveal the overall organization (Fig. 1B). Throughout the region analyzed, the L^d gene has exactly the same sequence arrangement as the K^d gene, except for the interruption by the NC2^L segment located at precisely the junction between NC1 and NC2^K. The 3' flanking sequence (FL3') immediately downstream of the NC2^K remained highly conserved in the two genes. The sequence of the corresponding region of the previously isolated D^d gene (10) was also determined and was identical in sequence organization to the L^d gene (Fig. 2).

The NC2^L sequence is highly repetitive in the mouse genome (11, 12) and is equivalent to the interspersed type 2 Alu-like repeated sequences (referred to as the B2 SINE sequence in mouse) that have been found in several rodents (13). All the Alu-like elements, including the type 2 sequences, are not only flanked by short direct repeats but are also bordered on the 3' side by an A-rich (A, adenine) region. In addition, they all contain putative RNA polymerase III promoters and termination signals (13).

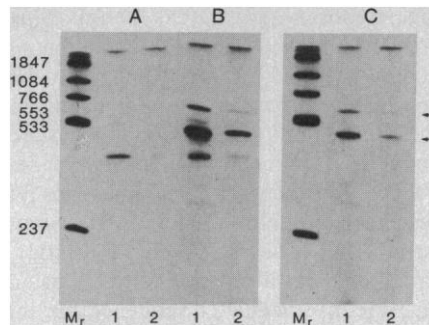


Fig. 3. S1 nuclease mapping of the 3' ends of the L^d transcripts. Polyadenylated RNA's from three lines of mouse cells, L cells (panel A), T1.1 cells (panel B), and BALB/c 3T3 cells (panel C) were separately hybridized with a single-stranded ³²P-labeled DNA probe derived from the 672-bp Pvu II fragment of λ L^dD5. For each RNA preparation, either 1 µg (lanes 1) or 0.25 µg (lanes 2) were used to ensure that hybridization was performed with an excess of DNA probe. The resulting DNA-RNA hybrids were treated with S1 nuclease and analyzed on a 6 percent polyacrylamide-urea gel with appropriate molecular weight markers (M_r). The arrows indicate DNA fragments that are ~620 and ~470 nucleotides in length.

The 174-bp NC2^L sequence is no exception. Apart from a short deletion of 11 nucleotides, it is more than 96 percent homologous to the consensus mouse B2 sequence (14) (Fig. 2). The putative split promoter for RNA polymerase III transcription (15) is located at nucleotide positions 323 to 333 and 356 to 367 from the termination codon of the L^d gene. The consensus termination signal, TTCTTT (15) (T, thymine; C, cytosine) is found just 5' to the polyadenylated tract at positions 469 to 474. The possibility that type 2 Alu-like sequences can function as transposable elements has been inferred from the presence of one such sequence at a specific region in the genome of only one mouse strain (16).

Distinct to the type 2 Alu-like repeats are polyadenylation signals (AATAAA) (A, alanine) located just in front of the recognition sequence for RNA polymerase III termination. In fact, in most of these sequences the polyadenylation signal is repeated several times and all are aligned in an overlapping array. In the NC2^L sequence, however, only one of these overlapping polyadenylation signals is conserved (Fig. 2). Since this signal is downstream from and contiguous with the coding exons, it may be functional. From the analysis of cDNA sequences derived from the L^d gene, this polyadenylation site seems to be preferentially used (17).

To test whether the presence of the NC2^L sequence could have preempted an otherwise functional polyadenylation

signal located near the end of the NC2^K region of the L^d gene, S1 nuclease mapping was performed (18). Cytoplasmic RNA from either control L cells or from a clone of L cells (T1.1.1) that stably expresses the transfected L^d gene (19) was hybridized with an excess of a ³²P-labeled single-stranded DNA probe derived from the 672-bp Pvu II fragment of the L^d gene (Fig. 1). Hybrids of RNA molecules with a polyadenylation signal at the end of the NC2^L region would yield an S1 fragment of 475 bp, and hybrids of molecules with a similar signal at the end of the NC2^K region further downstream would generate a fragment of 627 bp (Fig. 1). Analysis of the RNA from clone T1.1.1 (Fig. 3B) revealed two extra S1-resistant components of ~470 bp and ~620 bp, which were not present in RNA from the control parental L cells (Fig. 3A). This observation is consistent with the use of polyadenylation signals within both NC2^L and NC2^K regions of the L^d gene. On the basis of the intensity of the bands, the use of the NC2^L signal is more than 20 times as prevalent as the use of the NC2^K signal. The two background components (~390 bp and ~540 bp) in both L cells and T1.1.1 cells most likely were derived from RNA molecules with analogous polyadenylation sites in the endogenous L^k gene (previously thought to be the D^k gene). These species appear smaller than expected, perhaps as a result of polymorphic substitutions within the NC1 region that differ from the analogous region of the L^d probe used (6).

To confirm that the use of these polyadenylation sites is not peculiar to the transfected L^d gene, we analyzed RNA from BALB/c 3T3 cells (H-2^d haplotype) which have an endogenous L^d gene (Fig. 3C) and obtained similar results. Although the exact ratio for the use of the two polyadenylation signals is somewhat different, the NC2^L signal remained predominant over the NC2^K signal. This ratio is not significantly altered on transformation of BALB/c 3T3 cells by SV40 (data not shown).

The consequences of there being a type 2 Alu-like repeat at the 3' ends of the L^d and D^d mRNA's, as compared to the K^d transcript which does not contain this sequence, require deliberation. It has been observed that the K antigen is expressed to a greater degree on the cell surface than the D and L antigens in the same cell (6). Whether the type 2 Alu-like sequence is responsible for this difference in surface expression is now a conjecture. If this is the case, the repeat sequence may participate in altering the expression or the stability of the respec-

tive class I transcripts. In particular, small RNA's generated apparently by RNA polymerase III transcription of type 2 Alu-like sequences have been detected (20) which may, directly or indirectly, interact with the analogous repeat sequence at the 3' ends of the D^d and L^d genes or their mRNA's to regulate their transcription, processing, stability, or translational efficiencies.

There has been a recent suggestion that a class I transcript derived from the Qa2,3/Tla region of the MHC may also contain the type 2 Alu-like (NC2^L) sequence at a position analogous to that in the D^d and L^d transcripts (21). However, in view of the criteria that have been suggested for the identification of class I sequences (6), including locus-specific substitutions in both the NC1 and the transmembrane regions, we suggest that this RNA transcript may not derive from the Qa2,3/Tla region (21) but might more likely derive from the previously identified D^d gene (10). If this conclusion is correct, however, we would have expected this RNA transcript to be expressed in normal cells rather than to be induced only upon transformation (22).

Our study demonstrates the presence of the type 2 Alu-like sequence (NC2^L) within the 3' noncoding regions of both the L^d and D^d genes, but not the K^d gene. This type 2 Alu-like sequence provides a novel polyadenylation signal which is efficiently used in transcriptional processing of these class I genes. This clearly demonstrates a biologically important function for the type 2 Alu-like sequence.

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Antidepressants Cause Lethal Disruption of Membrane Function in the Human Protozoan Parasite *Leishmania*

Abstract. The antidepressant compounds clomipramine and nitroimipramine were *cidal* to extracellular promastigotes of both human protozoan parasites *Leishmania donovani* and *Leishmania major*. Clomipramine also killed amastigotes of both species within murine macrophages with no apparent toxicity to the host cells. Further, amastigotes were more sensitive than promastigotes to clomipramine. Clomipramine (100 micromoles per liter or 0.2 nanomole per 1×10^6 cells) inhibited L-proline transport in promastigotes. Synergistic inhibition of L-proline transport was observed with clomipramine after addition of either of the ionophores valinomycin or nigericin. These observations suggest that the cytotoxic effects of clomipramine result from its disruption of the proton electrochemical gradient of the parasite surface membrane.

The parasitic trypanosomatid protozoan *Leishmania donovani* is the causative agent of human visceral leishmaniasis. This parasite has a digenetic life cycle, including an extracellular flagellated promastigote form within the alimentary tract of its insect vector and an obligate intracellular amastigote form within mammalian macrophages (1). The organism actively accumulates solutes such as L-proline and D-glucose via carrier-mediated transport systems (2, 3). Both of these transport systems are driven by a proton electrochemical gradient ($\Delta\mu_{H^+}$) across the parasite surface membrane (3). The presence of H⁺ adenosine triphosphatase activity in the parasite's surface membrane was suggested by both fine structure cytochemistry and the observation that transport (L-proline and D-glucose) was inhibited by N,N'-dicyclohexylcarbodiimide while the organisms retained high levels of cellular adenosine triphosphate (ATP) and normal respiration (3, 4). These functional characteristics of the *L. donovani* surface membrane are different from those of its mammalian host, and such differences might be exploited for therapeutic purposes.

The tricyclic antidepressant drugs imipramine and 3-chlorimipramine (clomipramine) are efficient inhibitors of serotonin uptake in mammalian cells (5).

Both of these compounds also act as uncouplers of oxidative phosphorylation and at high concentrations as inhibitors of H⁺ adenosinetriphosphatases in mammalian mitochondria (6). The latter observation prompted us to investigate the effect of these compounds on both the growth and membrane functions of *L. donovani*. For comparative purposes, the effects of these compounds were also assessed with *Leishmania major*, the agent of Old World human cutaneous leishmaniasis.

Promastigotes of a cloned strain of *L. donovani* (7) were maintained and grown in chemically defined RE IX medium (8). BALB/c mice were infected with promastigotes of a cloned strain of *L. major* (9). Amastigotes were isolated from the excised foot pads of these animals 4 to 5 weeks after infection. These amastigotes were allowed to transform into promastigotes in medium M199 containing 20 percent fetal calf serum and 25 mM Hepes at 26°C.

The effect of imipramine and its analogs on the growth of promastigotes was assessed. Parasite cultures were initiated at 3×10^6 cells per milliliter and when they reached 1×10^7 to 2×10^7 cells per milliliter, drugs were added at various concentrations. The number of promastigotes in each culture was then determined every 24 hours.