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Immunology

Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system

(expression library/surface antigen/cloning strategy/Tp44)

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ABSTRACT **CD28 (Tp44) is a human T-cell-specific homodimer surface protein that may participate in T-cell activation. We have isolated a cDNA clone encoding CD28 by a simple and highly efficient cloning strategy based on transient expression in COS cells. Central to this strategy is the use of an efficient method to prepare large plasmid cDNA libraries. The libraries are introduced into COS cells, where transient expression of surface antigen allows the isolation of cDNAs by way of monoclonal antibody binding. The CD28 cDNA encodes a highly glycosylated membrane protein with homology to the immunoglobulin superfamily and directs the production of a homodimer in transfected COS cells.**

Monoclonal antibodies recognizing three antigens, CD3 (T3), CD2 (T11), and CD28 (Tp44), cause human T cells to proliferate in the presence of phorbol esters (1–3). Whereas CD3 appears to be involved in transduction of the signal generated by antigen binding to the T-cell receptor (4, 5), the role of the CD2 and CD28 antigens in physiological proliferation is not at present understood. Exposure of T cells to anti-CD28 or interleukin 1 results in increased levels of cytoplasmic cGMP (6, 7), and either interleukin 1 or anti-CD28 antibody can substitute for monocytes in provoking proliferation of T cells exposed to agarose-bound anti-CD3 antibody (7, 8). However, the molecular weight, tissue distribution, and surface density of the interleukin 1 receptor (9) and CD28 antigen (10) are dissimilar. Moreover, anti-CD28 treatment, but not interleukin 1 treatment, allows proliferation of phorbol ester-stimulated T cells in the presence of dibutyryl cAMP (11).

We have described (12) a monoclonal antibody-based technique for enrichment of cDNAs encoding surface antigens. Here we describe a method of constructing plasmid expression libraries that allows the enrichment technique to be fully exploited. The method for making plasmid expression libraries may be of more general use for expression cloning, since, with the exception of some lymphokine cDNAs isolated by expression in COS cells (13–16), few cDNAs in general have been isolated from mammalian expression libraries. There appear to be two principal reasons for this. (*i*) The existing technology (17) for construction of large plasmid libraries is difficult to master, and the library size, even in the hands of virtuosos, rarely approaches that accessible by phage cloning techniques (18). (*ii*) The existing vectors are, with one exception (13), poorly adapted for high-level expression, particularly in COS cells. The reported successes with lymphokine cDNAs do not imply a general fitness of the methods used, since these cDNAs are particularly easy to isolate from expression libraries. Lymphokine bioassays are very sensitive (13–16), and the mRNAs are typically both abundant and short (13–16).

In this article we have applied the antibody selection technique to isolate a cDNA clone encoding the CD28 antigen.* The antigen shares substantial homology with members of the immunoglobulin superfamily and forms a dimer structure on the surface of transfected COS cells similar to the dimer structure found on T lymphocytes.

MATERIALS AND METHODS

Preparation of cDNA Libraries. Poly(A)$^+$ RNA was prepared from the human T-cell tumor line HPB-ALL by oligo(dT)-cellulose chromatography of total RNA isolated by the guanidine thiocyanate method (19). cDNA was prepared by the following protocol, based on the method of Gubler and Hoffman (20). mRNA (4 μg) was heated to $\approx$100°C in a 1.5-ml centrifuge tube for 30 sec and quenched on ice, and the volume was adjusted to 70 μl with RNase-free water. To this were added 20 μl of buffer [0.25 M Tris, pH 8.8 (pH 8.2 at 42°C)/0.25 M KCl/30 mM $MgCl_2$], 2 μl of RNase inhibitor (Boehringer Mannheim; 36 units/μl), 1 μl of 1 M dithiothreitol, 1 μl of oligo(dT) at 5 μg/μl (Collaborative Research), 2 μl of each deoxynucleoside triphosphate at 25 mM (United States Biochemicals, Cleveland), and 4 μl of reverse transcriptase (Life Sciences, St. Petersburg, FL; 24 units/μl). After 40 min at 42°C the reaction was terminated by heating to 70°C for 10 min. To the reaction mixture was then added a solution containing 320 μl of RNase-free water, 80 μl of buffer [containing 0.1 M Tris·HCl (pH 7.5), 25 mM $MgCl_2$, 0.5 M KCl, bovine serum albumin at 0.25 mg/ml, and 50 mM dithiothreitol], 25 units of DNA polymerase I (Boehringer Mannheim), and 4 units of RNase H (Bethesda Research Laboratories). After 1 hr at 15°C and 1 hr at 22°C, 20 μl of 0.5 M EDTA (pH 8.0) was added, the reaction mixture was extracted with phenol, NaCl was added to 0.5 M, linear polyacrylamide (carrier; ref. 21) was added to 20 μg/ml, and the tube was filled with ethanol. After centrifugation for 2–3 min at 12,000 $\times$ *g*, the tube was removed, Vortex mixed to dislodge precipitate spread on the wall of the tube, and centrifuged again for 1 min.

Unpurified oligonucleotides having the sequence CTCTAAAG and CTTTAGAGCACA were dissolved in H_2O at a concentration of 1 mg/ml, $MgSO_4$ was added to 10 mM, and the DNA was precipitated by adding 5 volumes of EtOH. The pellet was rinsed with 70% (vol/vol) EtOH and resuspended in TE buffer [10 mM Tris·HCl (ph 7.5)/0.5 mM EDTA] at a concentration of 1 mg/ml. The resuspended oligonucleotides (25 μl) were phosphorylated by the addition of 3 μl of buffer [containing 0.5 M Tris·HCl (pH 7.5), 10 mM ATP, 20 mM dithiothreitol, 10 mM spermidine, bovine serum albumin at 1 mg/ml, and 10 mM $MgCl_2$] and 20 units of polynucleotide kinase followed by incubation at 37°C overnight.

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*This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02988).

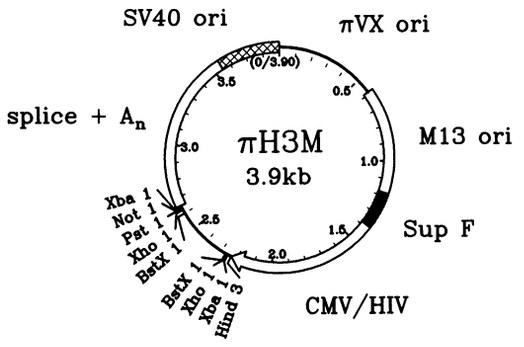

FIG. 1. Map of the πH3M vector. The direction of transcription is indicated by an arrow. Restriction endonuclease sites flanking the *Bst*XI cloning sites are shown. There are seven segments. Residues 1–587 are from the pBR322 origin (ori) of replication, residues 588–1182 are from the M13 origin, residues 1183–1384 are from the *supF* gene, residues 1385–2238 are from the chimeric cytomegalovirus/human immunodeficiency virus promoter, residues 2239–2647 are from the replaceable fragment, residues 2648–3547 are from plasmid pSV2 (splice and polyadenylylation signals), and residues 3548–3900 are from the simian virus 40 origin (SV40 ori). The complete nucleotide sequence is available from the authors.

The 12-mer (3 μl) and the 8-mer (2 μl) phosphorylated oligonucleotides were added to the cDNA prepared as above in a 300-μl reaction mixture containing 6 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, 5 mM NaCl, bovine serum albumin at 0.35 mg/ml, 7 mM 2-mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, 1 mM spermidine, and 400 units of T4 DNA ligase (New England BioLabs) at 15°C overnight. EDTA (10 μl at 0.5 M) was added; and the reaction was phenol extracted, ethanol precipitated, resuspended in a volume of 100 μl of TE, and layered on a 5-ml gradient containing 5–20% (wt/vol) potassium acetate, 1 mM EDTA, and ethidium bromide at 1 μg/ml. The gradient was centrifuged 3 hr at 50,000 rpm (SW55 rotor) and fractionated manually, collecting three ≈0.5-ml fractions followed by six ≈0.25-ml fractions in microcentrifuge tubes by means of a butterfly infusion set inserted just above the curve of the tube. Linear polyacrylamide was added to 20 μg/ml, the tubes were filled with ethanol, chilled, centrifuged, Vortex mixed, and centrifuged again as above. The precipitate was washed with 70% (vol/vol) ethanol, dried, and resuspended in 10 μl. One microliter of the last six fractions was electrophoresed on a gel to determine which fractions to pool, and typically material <1 kilobase (kb) was discarded. Remaining fractions were pooled and ligated to the vector.

The complete sequence and derivation of the vector are available on request. The vector was prepared for cloning by digestion with *Bst*XI and by fractionation on 5–20% (wt/vol) potassium acetate gradients as described for the cDNA. The appropriate band was collected by syringe under 300-nm UV light and ethanol precipitated as above. cDNA and vector were titrated in test ligations. Usually 1–2 μg of purified vector was used for the cDNA from 4 μg of poly(A)$^+$ RNA. The ligation reaction mixtures were composed as described for the adaptor addition above. The ligation reaction mixtures were transformed into MC1061/p3 cells made competent by an unpublished protocol available from Michael Scott (Department of Neurology, University of California, San Francisco) (personal communication). The transformation efficiency for supercoiled vector was 3–5 × 10^8 colonies per μg.

Recovery and Characterization of the CD28 Clone. Screening of the library was carried out as described by Seed and Aruffo (12), using purified antibody 9.3 (DuPont) at a concentration of 1 μg/ml of the antibody buffer. The methods used for COS cell transfection, radioimmunoprecipitation, RNA and DNA blot hybridization, and DNA sequencing were all as described (12).

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                                                                                                    -18                (120)
AGACTCTCAGGCCTTGGCAGGTGCGTCTTTCAGTTCCCCTCACACTTCGGGTTCCTCGGGGAGGAGGGGCTGGAACCCTAGCCCATCGTCAGGACAAAGATGCTCAGGCTGCTCTTGGCT
                                                                                                    MetLeuArgLeuLeuLeuAla
                                        +1                                                                  +20            (240)
CTCAACTTATTCCCTTCAATTCAAGTAACAGGAAACAAGATTTTGGTGAAGCAGTCGCCCATGCTTGTAGCGTACGACAATGCGGTCAACCTTAGCTGCAAGTATTCCTACAATCTCTTC
LeuAsnLeuPheProSerIleGlnValThrGlyAsnLysIleLeuValLysGlnSerProMetLeuValAlaTyrAspAsnAlaValAsnLeuSerCysLysTyrSerTyrAsnLeuPhe
                                                                                                    ---CHO--- *
                                    +40                                                             +60                (360)
TCAAGGGAGTTCCGGGCATCCCTTCACAAAGGACTGGATAGTGCTGTGGAAGTCTGTGTTGTATATGGGAATTACTCCCAGCAGCTTCAGGTTTACTCAAAAACGGGGTTCAACTGTGAT
SerArgGluPheArgAlaSerLeuHisLysGlyLeuAspSerAlaValGluValCysValValTyrGlyAsnTyrSerGlnGlnLeuGlnValTyrSerLysThrGlyPheAsnCysAsp
                                                        *           ---CHO---                                       *
                                    +80                                                             +100               (480)
GGGAAATTGGGCAATGAATCAGTGACATTCTACCTCCAGAATTTGTATGTTAACCAAACAGATATTTACTTCTGCAAAATTGAAGTTATGTATCCTCCTCCTTACCTAGACAATGAGAAG
GlyLysLeuGlyAsnGluSerValThrPheTyrLeuGlnAsnLeuTyrValAsnGlnThrAspIleTyrPheCysLysIleGluValMetTyrProProProTyrLeuAspAsnGluLys
            ---CHO---                                   ---CHO---           *
                                    +120                                                            +140               (600)
AGCAATGGAACCATTATCCATGTGAAAGGGAAACACCTTTGTCCAAGTCCCCTATTTCCCGGACCTTCTAAGCCCTTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGC
SerAsnGlyThrIleIleHisValLysGlyLysHisLeuCysProSerProLeuPheProGlyProSerLysProPheTrpValLeuValValValGlyGlyValLeuAlaCysTyrSer
   ---CHO---                        *                                                                  ----------TM------
                                    +160                                                            +180               (720)
TTGCTAGTAACAGTGGCCTTTATTATTTTCTGGGTGAGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCGCCCCGGGCCCACCCGCAAGCATTACCAG
LeuLeuValThrValAlaPheIleIlePheTrpValArgSerLysArgSerArgLeuLeuHisSerAspTyrMetAsnMetThrProArgArgProGlyProThrArgLysHisTyrGln
------------------------------------
                                    +202                                                                                (840)
CCCTATGCCCCACCACGCGACTTCGCAGCCTATCGCTCCTGACACGGACGCCTATCCAGAAGCCAGCCGGCTGGCAGCCCCCATCTGCTCAATATCACTGCTCTGGATAGGAAATGACCG
ProTyrAlaProProArgAspPheAlaAlaTyrArgSerEnd
                                                                                                                        (960)
CCATCTCCAGCCGGCCACCTCAGCCCCTGTTGGGCCACCAATGCCAATTTTTCTCGAGTGACTAGACCAAATATCAAGATCATTTTGAGACTCTGAAATGAAGTAAAAGAGATTTCCTGT
                                                                                                                        (1080)
GACAGGCCAAGTCTTACAGTGCCATGGCCCACATTCCAACTTACCATGTACTTAGTGACTTGACTGAGAAGTTAGGGTAGAAAACAAAAAGGGAGTGGATTCTGGGAGCCTCTTCCCTTT
                                                                                                                        (1200)
CTCACTCACCTGCACATCTCAGTCAAGCAAAGTGTGGTATCCACAGACATTTTAGTTGCAGAAGAAAGGCTAGGAAATCATTCCTTTTGGTTAAATGGGTGTTTAATCTTTTGGTTAGTG
                                                                                                                        (1320)
GGTTAAACGGGGTAAGTTAGAGTAGGGGGAGGGATAGGAAGACATATTTAAAAACCATTAAAACACTGTCTCCCACTCATGAAATGAGCCACGTAGTTCCTATTTAATGCTGTTTTCCTT
                                                                                                                        (1440)
TAGTTTAGAAATACATAGACATTGTCTTTTATGAATTCTGATCATATTTAGTCATTTTGACCAAATGAGGGATTTGGTCAAATGAGGGATTCCCTCAAAGCAATATCAGGTAAACCAAGT
                                                                                                                        (1514)
TGCTTTCCTCACTCCCTGTCATGAGACTTCAGTGTTAATGTTCACAATATACTTTCGAAAGAATAAAATAGTTC
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FIG. 2. Nucleotide sequence of the CD28 cDNA. Nucleotide numbering is given in parentheses at right; amino acid numbering is above the sequence and not in parentheses. Locations of the potential sites for addition of asparagine-linked carbohydrate (CHO) are shown, as well as the predicted transmembrane (TM) sequence. The amino acid sequence is numbered from the projected cleavage site of the secretory signal sequence. The presumed polyadenylylation signal is shown boxed.

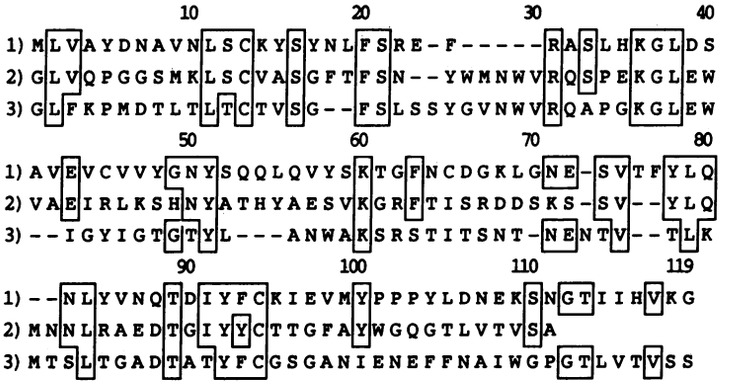

FIG. 3. Homology between the CD28 cDNA (lines 1), a mouse (lines 2), and a rabbit (lines 3) immunoglobulin heavy-chain variable region is shown boxed.

RESULTS AND DISCUSSION

To isolate the CD28 cDNA, a large plasmid cDNA library was constructed in a high-efficiency expression vector containing a simian virus 40 origin of replication. A version of the vector, containing an M13 origin, is shown in Fig. 1. Three features of the vector make it particularly suitable for this use. (*i*) The eukaryotic transcription unit allows high-level expression in COS cells of coding sequences placed under its control. (*ii*) The small size and particular arrangement of sequences in the plasmid permit high-level replication in COS cells. (*iii*) The presence of two identical *Bst*XI sites in inverted orientation and separated by a short replaceable fragment allows the use of an efficient oligonucleotide-based strategy to promote cDNA insertion in the vector.

The *Bst*XI cleavage site, $CCAN_5/NTGG$ (where the slash denotes the cleavage site), creates a 4-base 3′ extension that varies from site to site. We created a vector in which two identical sites were placed in inverted orientation with respect to each other and separated by a short replaceable segment of DNA. Digestion with *Bst*XI followed by removal of the replaceable segment yields a vector molecule that is capable of ligating to fragments having the same ends as the replaceable segment but not to itself. In parallel, we attached to the cDNA synthetic oligonucleotides that give the same termini as the replaceable segment. The cDNA then cannot ligate to itself but can ligate to the vector. In this way, cDNA and vector are used as efficiently as possible. Tailing with terminal transferase achieves the same end but with less convenience and, in our hands, less overall efficiency. Moreover, homopolymer tracts located on the 5′ side of the cDNA inserts have been reported to inhibit expression *in vitro* and *in vivo* (15, 22, 23). Similar approaches based on the use of partially filled restriction sites to favor insertion of genomic DNAs (24) and cDNAs (16) have been reported. These approaches give 2- or 3-base complementary termini, which usually ligate less efficiently than the 4-base extensions reported here.

Although our cloning scheme does not result in a directional insertion of the cDNA, the ability to make large libraries easily, coupled with a powerful selection procedure, makes directional insertion unnecessary. In preliminary studies considerable effort was devoted to developing an efficient bidirectional transcription unit that would allow either orientation to be expressed at high levels; but it appears that this goal cannot be easily attained in COS cells because of mutual interference arising when complementary transcripts are formed. The library construction efficiencies we observe, between 0.5 and 2×10^6 recombinants per μg of mRNA with <1% background and an insert size >1 kb, compare favorably with those described for phage vectors λgt10 (7.5×10^5 recombinants per μg of mRNA) and λgt11 (1.5×10^6 recombinants per μg of mRNA) (18); but the resulting clones are more convenient to manipulate.

Surface antigen cDNAs can be isolated from these libraries using an antibody-enrichment method (12). In this method, spheroplast fusion is used to introduce the library into COS cells, where it replicates and expresses its inserts. The cells are harvested by detaching without trypsin, treated with monoclonal antibodies specific for the surface antigens desired, and distributed in dishes coated with affinity-purified antibody to mouse immunoglobulins (12). Cells expressing surface antigen adhere, and the remaining cells can be washed away. [This general method of cell selection is known as "panning" (25)]. From the adherent cells, a "Hirt" fraction is prepared (26), and the resulting DNA is transformed back into *Escherichia coli* for further rounds of fusion and selection. Typically, after two rounds of selection with monoclonal antibodies recognizing various surface antigens, a single round of selection is performed with a single antibody or a pool of antibodies recognizing the same antigen (unpublished results).

Isolation of a CD28 cDNA. The CD28 cDNA was isolated from a library of $\approx 3 \times 10^5$ recombinants prepared from cDNA from 0.8 μg of poly(A)$^+$ RNA. The library was screened for CD28 (and other surface antigen) cDNA clones by the method outlined above (12). After the third transfection, COS cells were panned with the 9.3 antibody alone. A Hirt supernatant was prepared from the adherent cells and transformed into *E. coli*. Plasmid DNA was isolated from eight colonies and transfected individually into COS cell cultures. The presence of the CD28 antigen was detected in three of eight transfected cultures by indirect immunofluorescence. All three plasmid DNAs contained an insert of ≈1.5 kb.

cDNA Sequence Analysis. The CD28 cDNA encodes a long

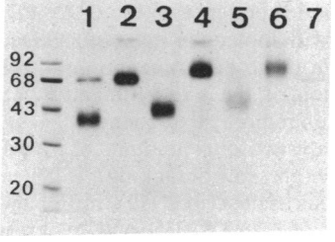

FIG. 4. Immunoprecipitation of CD28 antigen synthesized by T cells and transfected COS cells. Lanes: 1, 3, and 5, CD28 antigen from COS cells, activated T cells, and HPB-ALL cells, under reducing conditions; 2, 4, and 6, CD28 from COS cells, activated T cells, and HPB-ALL cells, under nonreducing conditions; 7, CD28-transfected COS cells treated with anti-CD4 antibody.

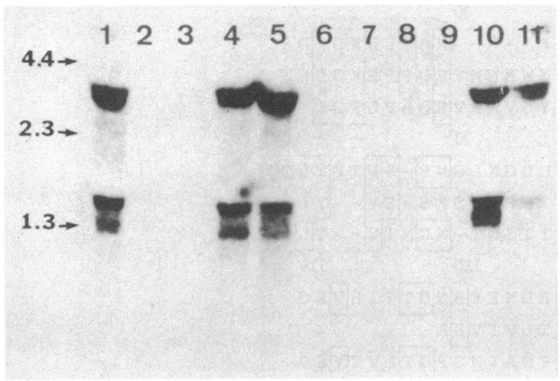

FIG. 5. RNA blot analysis of CD28-related transcripts. RNA sources were as follows. Lanes: 1, thymocytes; 2, U-937; 3, HuT 78; 4, T blast; 5, HPB-ALL; 6, Jurkat J3R7; 7, Namalwa; 8, MOLT-4; 9, HSB-2; 10, PEER; and 11, senescent (rested) T cells. RNA sizes (in kb) are from poly(A)$^+$ standards (Bethesda Research Laboratories).

open reading frame of 220 residues having the typical features of an integral membrane protein (Fig. 2). Removal of a predicted (27) N-terminal signal sequence gives a mature protein of 202 residues comprising an extracellular domain with five potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr), a 27-amino acid hydrophobic membrane-spanning domain, and a 41-amino acid cytoplasmic domain. Comparison of the amino acid sequence of CD28 with the National Biomedical Research Foundation Database† revealed substantial homology with mouse and rabbit immunoglobulin heavy-chain variable regions over a domain spanning almost the entire extracellular portion of CD28 (Fig. 3). Within this domain two cysteine residues in the homology blocks Leu-(Ser or Thr)-Cys and Tyr-(Tyr or Phe)-Cys are shared by CD28, CD4, CD8, immunoglobulin heavy- and light-chain variable sequences, and related molecules with approximately the same spacing (28–30).

CD28 cDNA Directs the Production of a Homodimer in Transfected COS Cells. Immunoprecipitation of CD28 antigen from transfected COS cells was carried out using the monoclonal antibody 9.3 (10). The material obtained from COS cells migrated with a molecular mass of 74 kDa under nonreducing conditions and 39 kDa under reducing conditions (Fig. 4), a pattern consistent with homodimer formation. Under the same conditions activated T cells give bands with molecular masses of 87 and 44 kDa, and HPB-ALL cells give bands of 92 and 50 kDa, under nonreducing and reducing conditions, respectively. The variation in molecular mass of the material obtained from various cell types likely arises as a result of differing glycosylation patterns characteristic of each type. We have observed similar results with other leukocyte surface antigens (B.S., unpublished results and ref. 12). The nucleotide sequence of the CD28 cDNA predicts a mature protein with molecular mass of 23 kDa, much smaller than observed in these experiments, and probably attributable to utilization of the five N-linked glycosylation sites predicted by the amino acid sequence.

RNA Blot Analysis. Equal amounts of total RNA prepared from cell types expressing or lacking CD28 were subjected to RNA blot analysis as described (12). Four bands (Fig. 5) with molecular sizes of 3.7, 3.5, 1.5, and 1.3 kb were visible in lanes containing RNA from thymocytes, T blasts, senescent T cells, and the T-cell leukemia cell lines PEER and HPB-ALL. No bands were detected in lanes containing RNA prepared from the cell lines U-937 (histiocytic leukemia), HuT 78 (adult T-cell leukemia), Jurkat (T-cell leukemia), Namalwa (Burkitt lymphoma), MOLT-4, and HSB-2, all of which do not express CD28. We presume that the 1.5-kb transcript corresponds to the isolated cDNA and that the 3.7- and 3.5-kb species reflect incomplete splicing or alternative polyadenylylation site utilization.

†Protein Identification Resource (1986) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 10.0.

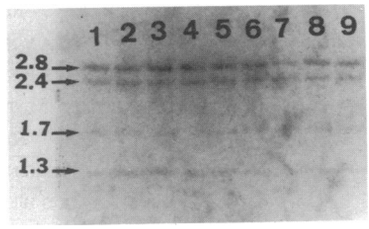

FIG. 6. Genomic DNA blot analysis. Genomic DNA (20 μg) digested with *Dra* I was electrophoresed through 0.8% agarose, blotted, and hybridized to a CD28 probe. DNA samples were prepared from the following cells. Lanes: 1, HuT 78 cells; 2, PEER cells; 3, MOLT-4 cells; 4, HSB-2 cells; 5, placenta; 6, Jurkat J3R7 cells; 7, total peripheral blood; 8, HPB-ALL cells; and 9, T blasts.

The CD28 Gene Is Not Rearranged. DNA blot analysis (12) of genomic DNA from placenta, peripheral blood lymphocytes, T cells, Hela cells, or the tumor lines used in the RNA blot analysis above showed identical *Dra* I digest patterns (Fig. 6), indicating that rearrangement is not involved in the normal expression of the CD28 gene during development. Similarly, no gross genomic rearrangement underlies the failure of the examined T-cell tumor lines to express CD28 antigen. We infer from the *Dra* I fragment pattern that the CD28 gene contains at least two introns.

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