1774–1779 *Nucleic Acids Research, 1996, Vol. 24, No. 9*

High-level production of recombinant proteins in CHO cells using a dicistronic DHFR intron expression vector

Brian K. Lucas, Lynne M. Giere, Richard A. DeMarco, Amy Shen, Vanessa Chisholm and Craig W. Crowley*

Department of Molecular Biology, Genentech, Inc., 460 Point San Bruno Boulevard, MS 60, South San Francisco, CA 94080-4990, USA

Received November 8, 1995; Revised and Accepted March 14, 1996

ABSTRACT

We have constructed expression vectors for Chinese hamster ovary (CHO) cells that produce both selectable marker and recombinant cDNA from a single primary transcript via differential splicing. These vectors produce stable CHO cell clones that, when pooled, produce abundant amounts of secreted recombinant proteins compared with the amounts produced by conventional expression approaches that have selectable marker and the cDNA of interest under control of separate transcription units. Our vectors divert most of the transcript to product expression while linking it, at a fixed ratio, to dihydrofolate reductase (DHFR) expression to allow selection of stable transfectants. Pools of clones with increased expression of the product gene can be efficiently generated by selection in methotrexate. The high level of expression from pools allows convenient and rapid production of milligram amounts of recombinant proteins.

INTRODUCTION

Production of recombinant proteins in mammalian cells has allowed the manufacture of a number of large, complex glycosylated polypeptides for clinical applications (1). Chinese hamster ovary (CHO) DHFR– cells (2) and the amplifiable selectable marker DHFR (3,4) are routinely used to establish cell lines that produce clinically useful amounts of product. However, stable expression of recombinant proteins in CHO and other mammalian cells is tedious and time consuming since only a small percentage of stable clones express large quantities of protein. Previous unpublished work has shown that expression levels of pooled, heterogeneously expressing clones are low, unstable, and cannot be appreciably increased by selection in methotrexate. This may be the result of rapid overgrowth by low or non-expressing cells.

We have developed a vector that can be used to generate stable clones that consistently express high levels of secreted recombinant proteins. The vector we describe in this report links expression of the selectable marker dihydrofolate reductase (DHFR) to the expression of the polypeptide of interest. This vector has been used to direct high level expression of a variety of recombinant proteins from pools of stable transfectants.

MATERIALS AND METHODS

Materials

CHO K1 DUX B11 (DHFR–) cells were obtained from L. Chasin (Columbia University). F12/DMEM medium and 2 mM L-glutamine was obtained from Gibco-BRL/Life Technologies (Gaithersberg, MD). Fetal bovine serum was from Hyclone (Logan, UT). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA). Rich medium (prepared at Genentech) is F12/DMEM based and contains high concentrations of amino acids and insulin (Nucellin, Eli Lilly, Indianapolis, IN). Amethopterin (MTX) was from Sigma (St Louis, MO). Monoclonal antibodies produced at Genentech, Inc. were used to quantify secreted tPA, TPO, anti-IgE and anti-CD11a antibodies using enzyme-linked immunoabsorbent assays (ELISA). Total RNA was prepared using standard methods (5), nylon filters were from Stratagene (La Jolla, CA), [^{35}S]Cys, [^{35}S]Met and [α-^{32}P]dCTP were from Amersham (Arlington Heights, IL).

Construction of plasmids

DHFR intron (DI) vectors were constructed by inserting the mouse DHFR cDNA into the intron of the expression vector pRK (6). The expression vector pRK is driven by the cytomegalovirus immediate-early gene promoter and enhancer (CMV IE P/E) and has a splice donor (SD) site derived from the cytomegalovirus immediate early gene (CMV IE) and a splice acceptor (SA) site from an IgG heavy chain variable region gene (7). An *Eco*RV site was inserted into a *Bst*XI site present 36 bases downstream of the splice donor (SD) site of the 144 bp intron of pRK. A 678 bp blunt ended fragment that contained the mouse DHFR cDNA (8) was inserted into the *Eco*RV site. Several different sequences were introduced into the existing SD site using an overlapping PCR strategy into a vector that contained a 2027 bp *Hin*dIII–*Cla*I tPA (9) encoding fragment. The tPA fragment had been inserted into the *Hin*dIII and *Cla*I sites of the cloning linker present 35 bases

* To whom correspondence should be addressed

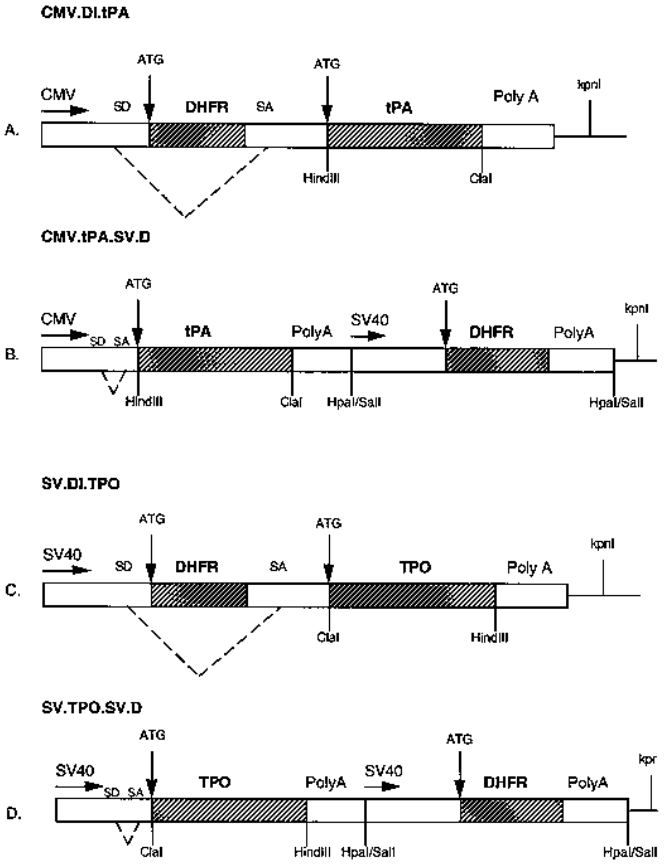

Figure 1. tPA and TPO DI and control expression vectors. (**A** and **C**) tPA and TPO DI vectors that direct expression of DHFR and product cDNAs via incomplete splicing of a single primary dicistronic RNA transcript. (**B** and **D**) Control tPA and TPO vectors that use separate expression units to produce DHFR and product.

downstream of the SA. The tPA vector (CMV.DI.tPA) that generated the most productive clones had the SD site GAC-GTAAGT and was used for subsequent studies. The control vector used in tPA experiments (CMV.tPA.SV.D) consists of the following elements: CMV IE P/E, the intron of pRK, the tPA cDNA, the SV40 polyA, the SV40 P/E, the DHFR cDNA and the polyA of Hepatitis B virus. The DHFR expression unit (*Sal*I cut and filled in) was inserted into an *Hpa*I site downstream of the tPA unit. The CMV IE P/E which was used for tPA expression was replaced with the SV40 P/E (10) using unique *Eag*I (located just 5′ of SD site) and *Kpn*I sites and was used to direct TPO (11) expression. The DI TPO (SV.DI.TPO) expression vector was constructed by inserting a 1112 bp *Cla*I–*Hin*dIII fragment encoding TPO into the DI vector using the same sites. The control TPO vector (SV.TPO.SV.D) is SV40 P/E driven, has the same 1112 bp TPO fragment and SV40 polyA as that found in the DI vector. The control vector has the same DHFR expression unit used for the tPA control vector. The vector that encoded the heavy chain of the anti-IgE antibody was constructed by inserting a *Bam*HI–*Cla*I ended cDNA fragment into a SV40 P/E driven DI vector. The anti-IgE (11) light chain/hygromycin vector was constructed by inserting an *Avr*II fragment that contained SV40 P/E, light chain and SV40 polyA sequence into an *Avr*II site downstream of the CMV/Hyg/polyA sequence in pRKHyg (13). Anti-CD11a antibody (14) expression was done using a single vector that expressed the heavy chain of the antibody as described for anti-IgE and expressed the light chain under control of a second SV40 P/E and used a second SV40 polyA. The light chain expression unit was inserted into an *Avr*II site downstream of the heavy chain expression unit.

Cell culture, transfections and selections

Prior to electroporation (15–17) CHO K1 DUX B11 cells were maintained in F12/DMEM medium supplemented with 10% FBS, L-glutamine (2 mM), glycine 10 μg/ml, hypoxanthine 15 μg/ml and thymidine 5 μg/ml (GHT). In experiments to express tPA, anti-IgE and anti-CD11a antibodies cells were trypsinized, washed, resuspended at 10^7/ml in 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM Dextrose, pH 7.05, mixed with 20 μg of linearized plasmid and 1 ml of cell suspension was pulsed using a BRL Cell-Porator at 800 μF, 300 V. Following electroporation, cells were transferred into 100 mM plates, grown for 2 days in nonselective medium, then fed with GHT free F12/DMEM with 7% dialyzed serum (DS) to select for DHFR+ clones. Expression of TPO was accomplished using lipofectamine (18) (Gibco-BRL) 20 μg of linearized plasmid was added to ~3×10^{-7} cells in 150 mm plates according to the manufacturer's instructions, selections were done as after electroporation. Seven to ten days after transfer to selective medium clones were isolated using sterile cotton swabs or pooled and transferred to wells in a 24-well plate to measure product titer. For DI and control TPO and tPA vector comparisons, stable pools were set up at 1×10^{-6} cells per 2 ml rich production media in 6-well plates in duplicate and the media was submitted for ELISA determination of product titer after 5 days incubation. For Anti-IgE antibody expression, cells were selected for DHFR expression and concurrently for hygromycin resistance using 200 μg/ml hygromycin B (Sigma). Antibody titers were determined in the same way except that the cells were incubated for 10 days before the medium was harvested for ELISA. Methotrexate selection of stable pools was accomplished by plating 5×10^5 cells into 100 mM plates containing GHT free F12/DMEM 7% DS and the indicated concentration of methotrexate.

RNA and protein analysis

RNA was extracted from 5×10^7 cells, 10 μg of total RNA was prepared and run on a 1.2% agarose, 6.6% formaldehyde gel, transferred to a nylon filter and probed with random primed probes prepared from isolated DNA fragments (Random Primers DNA Labeling System, Gibco-BRL). Anti-IgE antibody was analyzed by metabolically labeling confluent 35 mm dishes of cells with 50 μCi each of [^{35}S]Met and [^{35}S]Cys in Met/Cys-free medium. Cells were incubated in 2 ml labeling medium for 1 h, then 0.2 ml of serum free complete medium was added for 6 h. Ten μl of medium was run ± 2-mercaptoethanol reduction on 4–12% gradient SDS–PAGE gel, dried and exposed to film overnight.

RESULTS

Dicistronic and control expression vectors

The vectors we have constructed transcribe a dicistronic primary transcript which contains a mouse DHFR cDNA bounded by 5′ splice donor (SD) and 3′ splice acceptor (SA) intron splice sites, followed by the cDNA that encodes the protein of interest (Fig. 1A and C). These vectors are termed DI vectors. Full length message

produced by these vectors is translated to produce DHFR while spliced message produces recombinant product as it contains only that cistron. Several different SD sites were tested (data not shown) to identify one which would confer a DHFR+ phenotype to cells following transfection and which would divert the maximum amount of the transcript to production of recombinant protein. DI vectors which contained the cDNAs encoding the plasminogen activator tPA (9) (Fig. 1A) or the potent stimulator of megakaryocytopoiesis thrombopoietin (TPO) (10) (Fig. 1C) were linearized in the vector backbone before they were introduced into CHO cells. The control tPA expression vector is shown in Figure 1B and consists of CMV and SV40 promoter/enhancer (P/E) driven transcripts controlling tPA and DHFR respectively. The control vector for TPO experiments (Fig. 1D) contains separate SV40 P/E elements driving TPO and DHFR. Control vectors were also linearized prior to transfection.

Stable colony number and tPA titers produced by DI and control vectors

Figure 2 shows colony number and expression data obtained after transfection and selection of CHO DHFR– cells with CMV IE P/E driven conventional and DI tPA vectors. Figure 2A is a measure of the number of stable colonies that form after selection for a DHFR+ phenotype and gives an indirect measure of the extent to which DHFR expression has been weakened in the DI vector. DI vector derived clones, when pooled, and grown for 5 days produce 11-fold more tPA than that produced by a conventional vector derived pool (Fig. 2B). The weakening of DHFR expression and/or its linkage to product expression in the DI vector results in fewer but more productive clones when compared with the conventional vector.

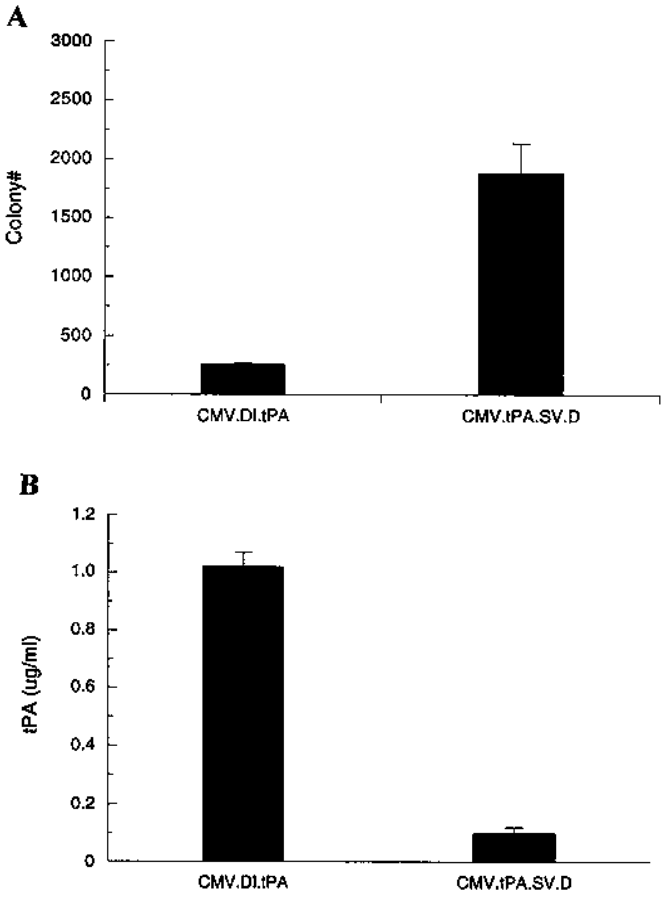

Figure 2. Colony number and tPA titers produced by DI and control tPA vector transfected cells. TPA expression vectors shown in Figure 1A and B were linearized and transfected by electroporation in duplicate into 1×10^7 CHO DHFR– cells and selected for stable DHFR expression. (**A**) Ten days after selection was begun stable colony number was determined. (**B**) Stable colonies were trypsinized and pooled, cultured for 5 days at which time the medium was collected and submitted for ELISA determination of tPA titer.

TPO titers and RNA products produced by DI and control vectors

The flexibility of the DI vector with regard to transcription initiation was tested by replacing the CMV IE P/E used to drive tPA expression with the comparable element from the early region of SV40 virus (14). SV40 and CMV IE P/Es have been shown to have similar high levels of transcriptional activity in CHO cells (unpublished observations). DI and control TPO expression vectors were constructed to direct the expression of TPO. Control and DI vectors were introduced into CHO cells by lipofection and were selected for a DHFR+ phenotype. Pools of the clones were made and tested for TPO productivity (Fig. 3A). Thirteen-fold more TPO is produced by the DI vector derived pool than by the conventional vector derived pool; in good agreement with data obtained when expressing tPA.

Highly productive TPO lines generated by DI and control vectors were also grown, lysed, and total RNA was subjected to Northern blot analysis. Figure 3B shows the RNA species present in DI, control vector transfected and non-transfected cells. The blot was probed sequentially with cDNAs encoding DHFR, TPO and β-actin. The DHFR probe detects an endogenous non-product producing RNA of ~2.3 kb from all three samples. The DHFR probe identifies the expected co-migrating RNA from DI.TPO transfected cells, and a product of 1.1 kb from cells transfected with the control TPO vector. The TPO probe hybridizes to the predicted 1.4 kb RNA product from both DI and control TPO vector transfected cells. Approximately 95% of the message produced by the DI vector is spliced and encodes only TPO. The remaining full length RNA is translated to produce DHFR to allow stable selection. Sequencing of cDNA made from mRNA produced by DI.TPO transfected cells confirmed that the RNA was spliced at the appropriate SD and SA sites (data not shown).

Production of antibodies using the DI vector

The utility of the DI vector system for antibody production was evaluated by expressing cDNAs encoding the heavy and light chains of humanized antibodies directed against IgE (12) and CD11a (manuscript in preparation). The anti-IgE antibody is capable of preventing IgE from binding to its receptor on cells and may be an effective inhibitor of allergic responses by blocking histamine release from mast cells (12). Anti-CD11a antibody binds CD11a on T cells and is capable of blocking the rejection of allografts in organ transplant models (manuscript in preparation).

Anti-IgE antibody production was achieved using an SV40 driven DI vector to drive heavy chain expression. The heavy chain vector was co-transfected with a second vector that contained the cDNA encoding the light chain of the antibody under control of the SV40 P/E and poly-A. The second vector also expressed the hygromycin B resistance gene under control of the CMV IE P/E.

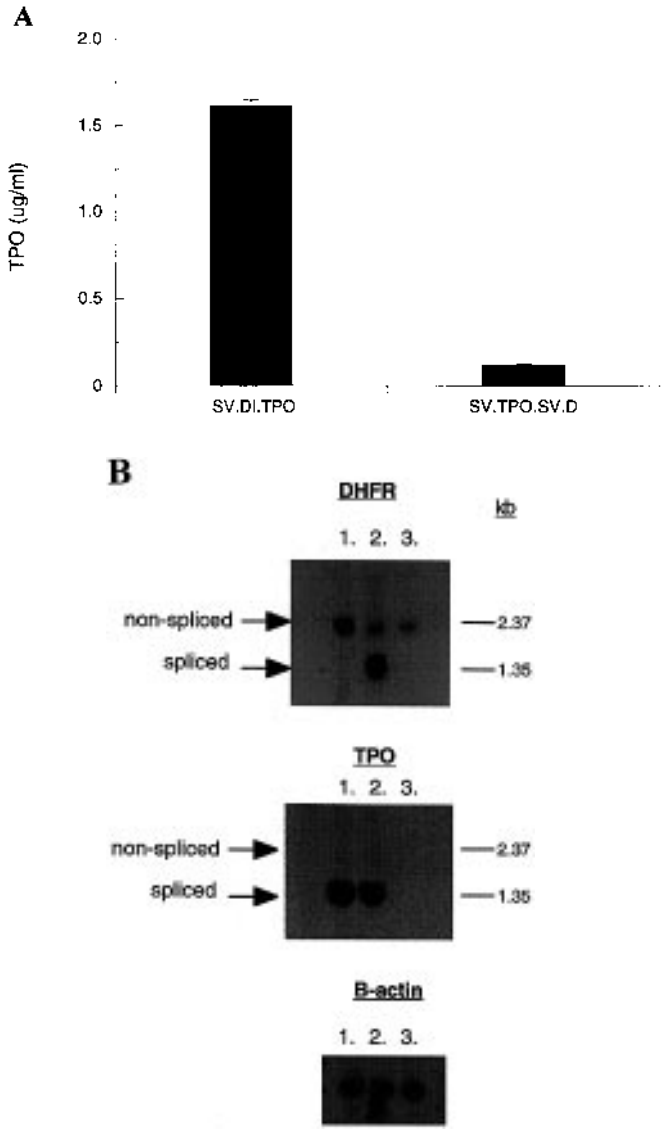

Figure 3. TPO titers and RNA products generated by DI and control vectors. The TPO expression vectors shown in Figure 1B and D were linearized and used to transfect by lipofection ~3×10^7 CHO DHFR– cells. (**A**) Colonies were selected for DHFR expression, pooled, and assayed for product accumulation after 5 days incubation by ELISA. (**B**) Northern blot analysis was preformed using total RNA extracted from highly productive TPO cell lines that were generated by DI (lane 1) and control (lane 2) vectors. RNA was also prepared from nontransfected cells (lane 3). RNA was probed sequentially using DNA fragments encoding DHFR, TPO and β-actin.

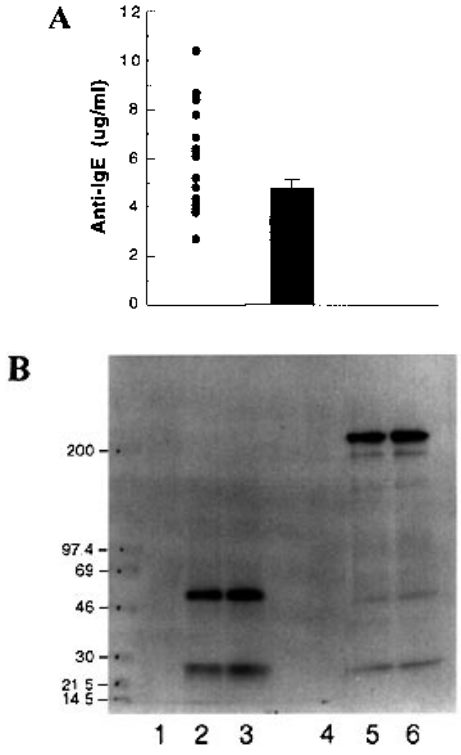

Figure 4. Anti-IgE antibody expression. Vectors encoding the heavy and light chains of the antibody are described in the text. CHO DHFR– cells were co-transfected with the vectors and selected for DHFR expression and hygromycin B resistance. The productivity of individual clones (closed circles) and of duplicate pools (bar) was determined after 10 days incubation at high density (**A**). The secreted antibody produced by the pool and that produced by a well characterized production clone were compared by running ^{35}S-labeled proteins with and without reduction on 4–12% SDS–PAGE gel. Pool derived samples (lanes 2 and 5), clone derived samples (lanes 3 and 6), nontransfected cell derived samples (lanes 1 and 4). Reduced samples were run in lanes 1–3, nonreduced samples were run in lanes 4–6 (**B**).

These vectors were linearized, mixed at a ratio of light chain vector to heavy chain vector of 10:3 and introduced into CHO cells by electroporation. Figure 4A shows the levels of antibody expressed by clones and a pool after selection in hygromycin B and by selection for DHFR expression. The pool and all 20 of the clones analyzed produced high levels of antibody when grown in rich medium. The levels of antibody secreted by the clones varied by only a factor of four. For the purpose of establishing a production cell line a clone is often desired. The high efficiency of recombinant protein production afforded by this methodology allows highly productive clones to be easily identified.

Figure 4B shows SDS gel analysis that compares the anti-IgE antibody produced by the pool (lanes 2 and 5) with that made by a well characterized production clone (lanes 3 and 6) generated by conventional vectors. Cells were metabolically labeled with [^{35}S]cysteine and methionine. CHO control cells were also labeled (lanes 1 and 4). Following a chase secreted proteins were run non-reduced (lanes 4–6) and reduced (lanes 1–3) on SDS–PAGE. The majority of the antibody protein is secreted as expected with a molecular weight of ~155 kDa, consistent with a properly disulfide-linked antibody molecule with two light and two heavy chains. Upon reduction the molecular weight shifts to two approximately equally abundant proteins of 22.5 and 55 kDa. The protein generated from the pool is indistinguishable from the antibody made by a well characterized production clone, with no apparent increase of free heavy or light chain expressed by the pool.

Anti-CD11a antibody was produced using a single vector that had the cDNA encoding the heavy chain of the antibody inserted in a DI expression unit and which also contained an SV40 driven light chain unit. Cells were transfected and selected for DHFR expression in the presence and absence of methotrexate at the concentrations indicated in Figure 5. The data show that exposure of transfected cells to methotrexate greatly reduces the number of surviving colonies and results in an increase of ~9-fold in the titer of secreted antibody.

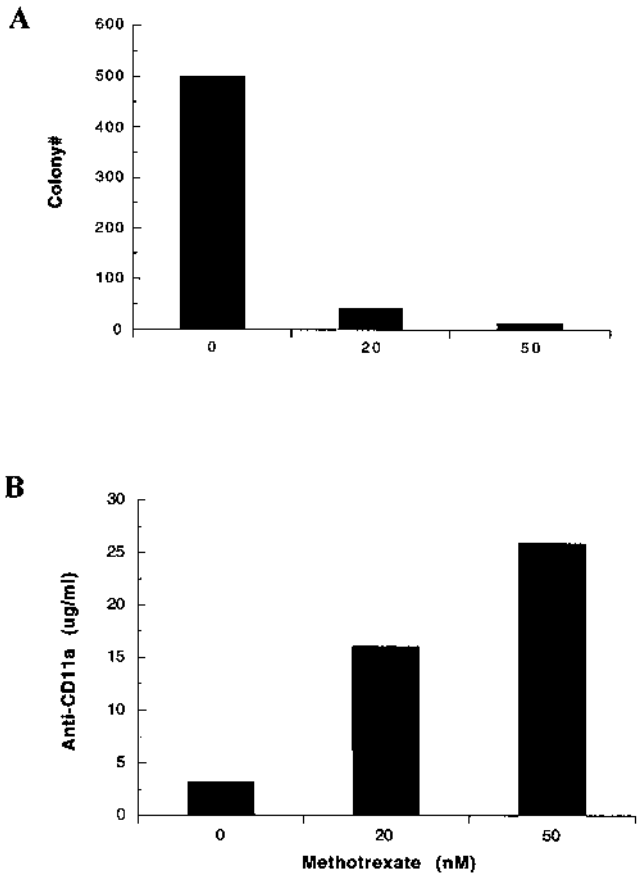

Figure 5. Colony number and anti-CD11a antibody titers obtained after transfection and selection in the presence and absence of methotrexate. Linear plasmid encoding both heavy and light chains of the antibody was transfected into 3×10^7 cells. Selections were done with methotrexate at the concentrations indicated, colonies were counted, then pooled and assayed by ELISA after 10 days incubation.

These pools have been maintained in monolayer or suspension culture for >60 days without loss of productivity demonstrating that they can be rapidly expanded for large scale production of antibodies (data not shown).

DISCUSSION

We have developed vectors for stable DHFR– CHO cell expression that produce high levels of recombinant proteins rapidly and with less effort than is required using conventional vectors. The vector has been optimized to produce high levels of recombinant product and a minimal amount of DHFR to allow selection of stable colonies. TPO and tPA are produced at 13- and 11-fold higher levels when DI vectors are used in place of conventional vectors. Intact antibodies have been produced efficiently using the DI vector strategy. The productivity of an anti-CD11a antibody expressing pool is stable in suspension culture for at least 60 days, allowing large scale fermentation runs, and its productivity is increased markedly with little effort by subjecting the pool to methotrexate selection. Antibody production from pools is very efficient even though the DI vector is used only to direct expression of the heavy chain of the antibody. The cDNA encoding the light chain is not transcriptionally linked to a selectable marker. It is possible that high level intact antibody production is achieved as cells that fail to produce light chain may be lost over time due to overgrowth by heavy and light chain positive cells as it has been shown that when heavy chain is produced alone in cells it is not secreted, is associated with BIP, and may be toxic at high levels (19).

The high productivity levels of pools produced by this vector may be due to product and DHFR linkage or because weakened DHFR function 'screens' for active site or multiple copy integration events. DHFR and product linkage is the likely explanation for the efficient increase in product titers when pools of DHFR-intron produced clones are selected for methotrexate resistance. Conventional vector derived pools contain an abundance of non-productive clones (data not shown) which probably are the result of breaks in the recombinant cDNA domain of the plasmid during integration into the genome or the result of methylation of promoter elements (20,21) driving expression of the product cDNA. Promoter silencing by methylation or breaks in the DI vectors would very likely render them incapable of conferring a DHFR+ phenotype.

Other mammalian expression vectors that produce two gene products from a single transcript have been described. Retroviral vectors have been constructed (22) in which a cDNA was inserted between the endogenous M-MULV SD and SA splice sites which are followed by a neomycin resistance gene. This vector has been used to express a variety of gene products following retroviral infection. It has not been optimized with regard to SD function and does not contain an amplifiable selectable marker. Other dicistronic vectors rely for colony formation on initiation of translation from a selectable marker open reading frame that is positioned downstream of the product reading frame (23,24). The utility of these vectors is variable because of the unpredictable influence of the upstream product reading frame on selectable marker translation and because the upstream reading frame may be deleted during methotrexate amplification (3). Later vectors (25–27) incorporated an internal ribosome entry site (IRES) derived from members of the picornavirus family which is positioned between the product and the downstream selectable marker coding regions. These vectors usually function quite efficiently, but in some cases selectable marker expression is adversely affected by the gene positioned upstream of the IRES (our unpublished observations). Other vectors have been constructed that place the cDNA of interest within an intron of a DHFR minigene (28). This vector helps to insure the integrity of the integrated construct, but transcriptional linkage is not achieved as DHFR and the cDNA of interest are driven by separate promoters.

The DI vectors we have constructed combine the advantages of previous dicistronic vectors as they link expression of DHFR and the cDNA of interest, may bias for active integration events and can accommodate a variety of cDNAs without the need for modification.

ACKNOWLEDGEMENTS

We thank Arthur Levinson and Justus Cohen for helpful discussions during the early part of this work. We also thank Paul Godowski for his suggestions regarding the work and for reviewing the manuscript.

REFERENCES

1 Levinson, A. D. and Goeddel, D. V. e. (1990) *Methods Enzymol.*, **185,** 485–487.
2 Urlab, G. and Chasin, L. A. (1980) *Proc. Natl Acad. Sci. USA*, **77,** 4216–4220.
3 Kaufman, R. J. and Sharp, P. (1982) *J. Mol. Biol.*, **159,** 601–621.

4 Gasser, C. S., Simonsen, C. S., Schilling, J. W. and Schmike, R. T. (1982) *Proc. Natl Sci. USA*, **79,** 6522–6526.
5 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual* (2nd Ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
6 Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J. and Martin, T. J. (1987) *Science*, **237,** 893–896.
7 Eaton, D. L., Wood, W. I., Eaton, D., Hass, P. E., Hollingshead, P., Wion, K., Mather, J., Vehar, G. A. and Gorman, C. (1986) *Biochem.*, **25,** 8343–8347.
8 Simonsen, C. C. and Levinson, A. D. (1983) *Proc. Natl Acad. Sci USA*, **80,** 2495–2499.
9 Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Yelverton, Y. F., Seeburg, P. H., Heyneker, H. L., Goeddel, D. V. and Collen, D. (1983) *Nature*, **301,** 214–221.
10 Griffin, B. E. (1980) In Tooze, J. (ed.) *DNA Tumor Virus.* Cold Spring Harbor, New York**,** pp. 61–124.
11 DeSauvage, F., Hass, P., Spencer, S.D., Malloy, B., Gurney, A., Spencer, S.A., Darbonne, W., Henzel, W., Wong, S., Kuang, W., Oles, K., Hultgren, B., Solberg, L., Goeddel, D., and Eaton, D. (1994) *Nature*, **369,** 533–538.
12 Presta, L. G., Lahr, S. J., Sheilds, R. L., Porter, J. P., Gorman, C. M., Fendly, B. M. and Jardieu, P. M. (1993) *J. Immunol.*, **151,** 2623–2632.
13 Yates, J. L., Warren, N. and Sugden, B. (1985) *Nature*, **313,** 812.
14 Manuscript in preparation.
15 Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofchneider, P. H. (1982) *EMBO J.*, **1,** 841–845.
16 Potter, H., Weir, L. and Leder, P. (1984) *Proc. Natl Acad. Sci. USA*, **81,** 7161–7165.
17 Chu, G., Hayakawa, H. and Berg, P. (1987) *Nucleic Acids Res.*, **15,** 1311–1326.
18 Hawley-Nelson, P., Ciccarone, V., Gebeyehu, G., Jesse, J. and Felgner, P. L. (1993) *Focus*, **15**(73).
19 Hendershot, L., Bole, D. and Kearney, J. F. (1987) *Immunol. Today*, **8,** 111–114.
20 Busslinger, M., Hurst, J. and Flavell, R. A. (1983) *Cell*, **34,** 197–206.
21 Watt, F. and Molloy, P. L. (1988) *Genes Dev.*, **2,** 1136–1143.
22 Cepko, C. L., Roberts, B. E. and Mulligan, R. C. (1984) *Cell*, **37,** 1053–1062.
23 Liu, C. C., Simonsen, C. C. and Levinson, A. D. (1984) *Nature*, **309,** 82–85.
24 Kaufman, R. J., Murtha, P. and Davies, M. V. (1987) *EMBO J.*, **6,** 187–193.
25 Pelletier, J. and Sonenberg, N. (1988) *Nature*, **334,** 320–325.
26 Jang, S. K., Davies, M. V., Kaufman, R. J. and Wimmer, E. (1987) *J. Virol.*, **63,** 1651–1660.
27 Davies, M. V. and Kaufman, R. J. (1992) *J. Virol.*, **66,** 1924–1932.
28 Abrams, J. M., Thorpe, S. M. and Schimke, R. T. (1989) *J. Biol. Chem.*, **264,** 14016–14021.