The Responsiveness of Embryonic Stem Cells to Alpha and Beta Interferons Provides the Basis of an Inducible Expression System for Analysis of Developmental Control Genes

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Embryonic stem (ES) cells, derived from the inner cell mass of the preimplantation mouse embryo, are used increasingly as an experimental tool for the investigation of early mammalian development. The differentiation of these cells in vitro can be used as an assay for factors that regulate early developmental decisions in the embryo, while the effects of altered gene expression during early embryogenesis can be analyzed in chimeric mice generated from modified ES cells. The experimental versatility of ES cells would be significantly increased by the development of systems which allow precise control of heterologous gene expression. In this paper, we report that ES cells are responsive to alpha and beta interferons (IFNs). This property has been exploited for the development of inducible ES cell expression vectors, using the promoter of the human IFN-inducible gene, 6-16. The properties of these vectors have been analyzed in both transiently and stably transfected ES cells. Expression was minimal or absent in unstimulated ES cells, could be stimulated up to 100-fold by treatment of the cells with IFN, and increased in linear fashion with increasing levels of IFN. High levels of induced expression were maintained for extended periods of time in the continuous presence of the inducing signal or following a 12-h pulse with IFN. Treatment of ES cells with IFN did not affect their growth or differentiation in vitro or compromise their developmental potential. This combination of features makes the 6-16-based expression vectors suitable for the functional analysis of developmental control genes in ES cells.

Murine embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the preimplantation mouse embryo (5, 16). These cells, which can be cultured in the undifferentiated state in vitro for extended periods of time, retain the ability to participate normally in mouse development such that upon reintroduction into the mouse blastocyst, they can contribute differentiated progeny to all tissues of the mouse, including the germ line (3, 22). This implies that ES cells can both generate and respond to all signals that regulate normal murine development. The ability of ES cells to differentiate into a variety of cell types in vitro (23) can therefore be exploited as a model system for the identification of inter- and intracellular signals that regulate self-renewal and differentiation during early development.

ES cells can also be used as a vehicle for the introduction of transgenes into the early murine embryo. The consequences of ectopic gene expression on early developmental events can be assessed in chimeric animals generated by blastocyst injection of genetically modified ES cells (2, 29).

The development of improved systems for controlled and reproducible expression of transgenes in ES cells would offer new opportunities for the identification and characterization of gene products that regulate developmental decisions in mammals. In particular, an ability to establish stable transfectants in the absence of transgene expression would allow access to factors which promote differentiation or cell death or which otherwise inhibit stem cell proliferation. The requirement for an inducible expression system which is essentially inactive in the absence of the specific inducer precludes the use of commonly available systems, such as

those based on the metallothionein promoter (9), which have a significant level of uninduced expression in ES cells (unpublished observations). In this report, we demonstrate that ES cells are responsive to alpha and beta interferons (IFN- α and - β) and that this property can be exploited for the construction of an inducible expression system based on the regulatory elements of the human IFN-inducible gene 6-16.

MATERIALS AND METHODS

Vector construction. Plasmid pXMT2 is a modified version of the COS cell expression vector pMT2 (12) containing the restriction enzyme sites *Pst*I, *Kpn*I, *Xho*I, *Xho*I, *Kpn*I, *Pst*I, and *Eco*RI inserted between the unique *Pst*I and *Eco*RI sites in pMT2. Plasmid pOT was constructed by ligation of the 2.6-kb *Eco*RI-*Bgl*II fragment of pBLCAT3 (15) and the 1.6-kb *Eco*RI-*Bgl*II fragment of pSV2- β globin (7). This vector therefore consists of a polylinker upstream of the simian virus 40 small-t intron and polyadenylation sequences.

Human 6-16 promoter fragments were obtained from pSV0CAT-based plasmids described by Porter et al. (19). 6-16 fragment 1 (1,040 bp) extends from the *Bgl*II site at -603 to the *Bam*HI site at +437 in intron 1. This was obtained as a *Bgl*II-*Sma*I fragment (19), end filled; and ligated into *Xba*I-digested, end-filled pOT to produce p1OT. 6-16 fragment 3 (645 bp), which extends from the *Bgl*II site at -603 to the *Nci*I site at +42 in exon 1, was obtained as a *Hind*III-*Nci*I fragment (19), end filled, and ligated to *Xba*I-digested, end-filled pOT to produce p3OT. 6-16 fragments 1 and 3 were isolated from p1OT and p3OT by *Hind*III-*Bgl*II digestion and ligated into *Hind*III-*Bgl*II-digested pXMT2 to produce the vectors p1OX and p3OX, respectively.

The promoterless bacterial chloramphenicol acetyltrans-

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ferase (CAT) gene from pBLCAT3 (15) was isolated as a *Bsr*YI fragment and ligated into *Bgl*II-digested expression vectors to produce the CAT expression vectors p1CX, p1CT, p3CX, and p3CT.

Plasmid pn6-16DIA was constructed by insertion of the coding sequence for the diffusible form of human differentiation-inhibiting activity/leukemia-inhibitory factor (DIA/LIF) from plasmid pC10-6R (27) into the *Bgl*II site downstream of the 6-16 sequences in p1OT. The vector also carries the neomycin phosphotransferase resistance (*neo*) cassette ppgkneopa, derived from pPNT (28), inserted into the unique *Eco*RI site in p1OT in the opposite transcriptional orientation to the DIA/LIF transcription unit. Digestion with *Eco*RI linearizes the plasmid and leaves the DIA/LIF and *neo* expression cassettes separated by the plasmid backbone.

Plasmid DNA was purified by CsCl density centrifugation. All DNA manipulations were carried out as described by Sambrook et al. (24), using enzymes from Boehringer Mannheim and Bresatec.

Cell culture and assays. CP1 (3), E14TG2a (10), EFC-1 (17), and MBL-5 (18) ES cells have been described elsewhere. CGR-2 ES cells were established from strain 129 mice by established procedures (23). ES cells were maintained in the absence of feeder cells essentially as described previously (26).

Transfections of the CAT expression plasmids were carried out by a modification of the method described by Chen and Okayama (4). Exponentially growing ES cells were seeded at a density of 5×10^5 cells per 9.5-cm² well and grown for 24 h at 37°C under 10% CO₂. Precipitates were formed with 5 µg of DNA mixed with 125 µl of 0.25 M CaCl₂ and 125 µl of 2× BBS (pH 6.99). Cells were transfected for 16 h under 3% CO₂ in a 50:50 mix of Dulbecco's modified Eagle's medium and Ham's F12 (26) containing 10% fetal calf serum. The precipitate was washed from the cells, and the cells were incubated under standard culture conditions. Cells were treated with a mixture of mouse IFN-α and -β (4.7 × 10⁵ IU/ml; Sigma) 2 h after the precipitate was removed from the cells. Transiently transfected cells were cotransfected with a β-galactosidase expression plasmid pCHI10 (Pharmacia) at a ratio of 4:1. β-Galactosidase expression was determined by the method of Hall et al. (8) and used to normalize transient transfection efficiencies. Stable transfectants were produced by cotransfection of the expression vector and the Neo^r expression plasmid pPGKneoβ (20) at a ratio of 5:1 and were selected with 200 µg of G418 (GIBCO) per ml. CAT assays were carried out by the method of Gorman et al. (7) and quantitated by liquid scintillation counting. Induction ratios were calculated as percent chloramphenicol conversion in the presence of IFN/percent chloramphenicol conversion in the absence of IFN.

Transfection of pn6-16DIA DNA was carried out by electroporation at 800 mV and 3 µF in a Bio-Rad gene pulser, using 50 µg of linearized DNA and 10⁷ EFC-1 ES cells. Preparation of cytoplasmic mRNA and Northern (RNA) hybridization analysis were carried out as described previously (21). The human DIA/LIF antisense RNA probe was prepared by in vitro transcription of HpGEM1 (20) with T7 polymerase.

Production of chimeric mice. Chimeric mice were produced by established procedures (23), using CGR-2 and E14TG2a ES cells and C57/B16 host blastocysts. Both CGR-2 and E14TG2a ES cells are homozygous *c^{ch}/c^{ch}* so that chimerism was readily detected by the presence of sandy coat color pigmentation. Male chimeras were mated

with outbred MF1 albino females to test for germ line transmission of the ES genotype revealed by the production of chinchilla pups.

RESULTS

Dose-responsive inducible gene expression in transiently transfected ES cells. Two fragments of the human 6-16 promoter were used to construct IFN-inducible expression vectors (Fig. 1). The activities of these promoter fragments in ES cells and their responsiveness to IFN-α and -β were initially investigated in transient assays using CAT as a reporter gene in two different plasmid backbones. Each of the 6-16-based expression vectors directed IFN-inducible expression in transiently transfected ES cells (Fig. 2A). With IFN at 500 IU/ml, levels of induction between 3.1-fold (p3CT) and 46.6-fold (p1CX) were achieved, although absolute induction ratios are difficult to assess when uninduced levels of expression are extremely low. This result establishes for the first time that ES cells are responsive to IFN-α and -β and must therefore express the receptors and signaling molecules that direct the transcriptional response to IFN. Vectors containing the longer 6-16 fragment 1 (p1CX and p1CT) directed higher levels of induced expression than did equivalent vectors based on fragment 3 (p3CX and p3CT). This finding is consistent with previous observations on differentiated cell lines, including T98G neuroblastoma cells (6), HeLa cells (13), U937 macrophages, F2001 and MRC5 fibroblasts (14), and Ltk⁻ cells (13). The levels of expression directed by both 6-16 promoters in the vectors based on pXMT2 (p1CX and p3CX) were higher than the levels directed by the promoters in the vectors based on pOT (p1CT and p3CT). This resulted in 7.5- to 9-fold-higher absolute levels of induced expression and in higher induction ratios. p1CX had the highest absolute levels of induced expression, but this was offset by the marginally higher basal-level expression directed by this vector in the absence of IFN.

All plasmids exhibited a linear increase in the levels of CAT expression in response to increasing levels of IFN between 0 and 1,000 IU/ml. Representative data are shown for p1CX in Fig. 2B and C. Precise control over the level of heterologous gene expression in transiently transfected ES cells could therefore be achieved by induction with the appropriate dose of IFN.

Dose-responsive inducible expression in stably transfected ES cells. Stably transfected MBL-5 ES cell lines containing the 6-16 expression vectors p1CX (Fig. 3) and p3CT (results not shown) were screened for CAT expression before and after treatment with IFN. IFN-inducible expression was observed in nine of nine independent p1CX and nine of nine independent p3CT cell lines. In each case, CAT expression could not be detected in the absence of IFN. This points to a very low level of basal activity of the 6-16 promoter in stably transfected ES cells, since the CAT assay can detect as little as 5 to 10 pg of CAT protein (25). The undetectable basal expression directed by p1CX in stably transfected ES cells contrasts with the low but detectable level observed in transiently transfected ES cells.

CAT expression in stably transfected cell lines was strongly stimulated by treatment of cells with IFN. Induction ratios of up to 100-fold could be achieved by stimulation of the cells with 500 IU of IFN per ml for 48 h (Fig. 3B). Induced expression in stable cell lines containing p1CX (Fig. 4) and p3CT (results not shown) increased linearly with increasing concentrations of IFN, as previously observed

FIG. 1. Schematic diagram of the construction of the IFN-inducible ES cell expression vectors. The 1,040-bp (A) and 645-bp (B) 6-16 promoters were inserted into the pXMT2 and pOT vectors to produce four IFN-inducible ES cell expression vectors (C and D). The CAT reporter gene was then inserted into each of the four vectors at the unique *Bgl*II site. ISRE, IFN-stimulable response element; T, TATA box; DHFR, murine dihydrofolate reductase gene; VAI, adenovirus VAI gene, SV40 polyA sequence, SV40 IVS, simian virus 40 small-t antigen intron; AMP^R, β -lactamase gene; ORI, *Escherichia coli* origin of replication; horizontal arrow, transcription start site. Restriction sites: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; Hc, *Hinc*II; N, *Nci*I; P, *Pst*I; S, *Sph*I; Sa, *Sal*I; X, *Xba*I.

with transiently transfected cells. Maximal induced expression was observed with about 1,000 IU of IFN per ml (Fig. 4C).

Time course of induced expression. The time course of IFN-induced expression was analyzed by using a stable MBL-5 ES cell line containing plasmid p1CX. CAT expression was monitored 0, 2, 6, 12, 24, and 48 h after cells had been treated with IFN at 1,000 IU/ml (Fig. 5). High levels of induced CAT activity were detectable 6 h after exposure of ES cells to IFN and were maintained for a further 42 h in the continuous presence of IFN (Fig. 5, filled squares). Removal of IFN from the culture medium after 8 h (Fig. 5, filled diamonds) or 10 h (Fig. 5, open squares) had little effect on the level of CAT expression at 24 h but resulted in a modest

decrease in the levels of expression seen at the 48-h time point.

IFN induction acts at the level of mRNA expression. Plasmid pn6-16DIA was constructed to investigate whether the 6-16 sequences could direct IFN-inducible expression of heterologous genes other than CAT. pn6-16DIA contains the coding sequence for the diffusible form of human DIA/LIF (27) inserted downstream of the 6-16 sequences in p1OT. The plasmid also contains a selectable *neo* cassette under the control of the murine phosphoglycerate kinase 1 (*pgk-1*) promoter (1). *Eco*RI-linearized pn6-16DIA was introduced into EFC-1 ES cells by electroporation, and G418-resistant colonies were isolated. After expansion of individual clones, integration events were analyzed by Southern hybridization

FIG. 2. (A) IFN-inducible expression in transiently transfected MBL-5 ES cells. ES cells were transiently cotransfected with the β -galactosidase expression vector pCH110 and expression vectors p1CX, p1CT, p3CX, p3CT, or pBLCAT3. Cells were grown in the continuous presence of 0 (lanes 1, 3, 5, 7, and 9) or 500 (lanes 2, 4, 6, and 8) IU of IFN per ml for 48 h. CAT assays were carried out with 0.075 U of β -galactosidase (β gal) activity. (B and C) Dose response of the IFN-inducible expression vector p1CX in transiently transfected MBL-5 ES cells. (B) CAT assays were carried out with 30 μ g of protein obtained from ES cells transiently cotransfected with p1CX and pCH110 after growth for 48 h in the continuous presence of 0 (lane 1), 50 (lane 2), 100 (lane 3), 250 (lane 4), 500 (lane 5), or 1,000 (lane 6) IU of IFN per ml. (C) Graphical representation of data shown in panel B.

(not shown). Four independent stable cell lines containing single inserts were subjected to mRNA analysis. Parallel cultures were incubated for 24 h in the presence or absence of 1000 IU of IFN per ml, and cytoplasmic RNA preparations were assayed for expression of the human DIA transgene by Northern hybridization (Fig. 6). In the absence of IFN, human DIA mRNA was not detectable in cell lines 1 and 4 and was present at extremely low levels in clones 2 and 3. All four clones showed similar, very high levels of transgene mRNA in response to IFN. This result indicates that the 6-16 system can be used to direct inducible expression of a range of heterologous genes, including mammalian cDNAs in ES cells, and that IFN induction is mediated largely, if not wholly, at the level of mRNA expression.

IFN treatment does not affect the developmental potential of ES cells. The response of MBL-5 and CP1 ES cells to IFN was assessed in clonal density cultures which were treated with increasing amounts of IFN. Concentrations of IFN up to 5,000 IU/ml had no discernible effect on colony number or colony size. Moreover, inhibition of differentiation was maintained in cultures supplemented with DIA/LIF,

whereas differentiation proceeded normally in cultures withdrawn from DIA/LIF. This finding indicates that IFN does not interfere with the processes of ES cell renewal or differentiation in vitro.

ES cells were also injected into blastocysts to investigate whether exposure to IFN had impaired their ability to contribute to chimeras. CGR-2 and E14TG2a ES cells were cultured in the presence of IFN at 500 and 1,000 IU/ml, respectively, for 24 h immediately prior to microinjection. In both cases, several overt coat color chimeras were obtained. These animals were healthy with no signs of any abnormality. The E14TG2a chimeras exhibited particularly extensive ES cell contributions, and one of these has now transmitted the ES genotype to viable offspring. This result demonstrates that pretreatment with IFN is compatible with chimera production and does not compromise the developmental potential of ES cells.

DISCUSSION

At the cellular level, developmental events such as self-renewal, differentiation, and programmed cell death are often critically dependent on the precise timing and level of gene expression. The analysis of these processes would therefore be significantly enhanced by the development of systems that allow the precise manipulation of gene expression. An ideal expression system for use in ES cells would exhibit low levels of uninduced expression (below the threshold required for biological activity), give a controllable range of induced expression levels, and use an inducing agent that does not compromise the pluripotential character of the cells. In this report, we describe the construction and characterization of an inducible expression system which fulfills these requirements in both transiently and stably transfected ES cells.

We have shown that ES cells are responsive to IFN- α and - β . Levels of expression directed by the IFN-inducible 6-16 promoter were extremely low or undetectable in the absence of IFN, could be stimulated up to 100-fold by treatment of cells with IFN, and increased in linear fashion in response to

FIG. 3. IFN-induced expression directed by p1CX in stable MBL-5 ES cell lines. (A) CAT assays were carried out with 35 μ g of protein obtained from two independent ES cell lines containing p1CX, i.e., p1CX 2.1 (lanes 1 and 2) and p1CX 2.6 (lanes 3 and 4) after growth for 48 h in the presence of 0 (lanes 1 and 3) or 500 (lanes 2 and 4) IU of IFN per ml. (B) Quantitation of results shown in panel A.

FIG. 4. Dose response of p1CX in stable MBL-5 ES cell line 3.9. CAT assays were carried out with 50 μ g of protein obtained from a stable ES cell line (p1CX3.9) containing p1CX after growth in the presence of 0 (lane 1), 50 (lane 2), 150 (lane 3), 250 (lane 4), 350 (lane 5), or 500 (lane 6) IU of IFN per ml for 48 h (A) or 0, 50, 100, 500, 1,000, or 5,000 IU of IFN per ml for 48 h (C). (B) Graphical representation of results presented in panel A. % Con CM, percent conversion of chloramphenicol to acetylated forms.

increasing levels of IFN. A single 6-16 vector could therefore be used to direct any required level of gene expression by manipulation of the dose of IFN used for induction. This precise refinement of expression levels is not available with other ES cell expression systems.

Although our analysis has been carried out primarily in MBL-5 ES cells, the 6-16 vectors also direct IFN-induced expression in independently isolated ES cell lines such as EFC-1, E14, and CP1. The 6-16 vectors are therefore likely to be generally useful for the regulated expression of transgenes in ES cell lines.

Two derivatives of the 6-16 promoter were tested. Higher levels of induced expression and higher induction ratios were directed by 6-16 fragment 1 (Fig. 1A). This is consistent with results obtained in terminally differentiated cells (19) and points to the existence of a positive signal for gene expression in the first intron of the 6-16 gene. Induced levels of expression for both promoters were higher in vectors derived from pXMT2 than in vectors derived from pOT. This influence of vector context on expression is significant, although its basis remains unclear. It may reflect a positive effect of VAI sequences on IFN-induced gene expression directed by pXMT2-based vectors (12) and/or aberrant pro-

cessing of the simian virus 40 small-t antigen intron (11) in the pOT-based vectors.

High levels of induced heterologous gene expression were detectable 6 h after IFN treatment and were maintained for at least 48 h both in the continuous presence of IFN and following IFN withdrawal after 8 or 10 h of induction. Induction occurred primarily at the level of RNA expression.

Treatment of ES cells with the inducing agent IFN did not induce differentiation or appreciable growth arrest even at concentrations of 5,000 IU/ml. ES cells treated with 1,000 IU of IFN per ml for 24 h retained the ability to participate normally in murine embryogenesis, indicating that the developmental potential of the cells had not been compromised.

The development of an effective inducible expression vector for use in murine ES cells has important implications for the use of these cells as an experimental system for the analysis of mammalian development. Factors that regulate developmental decisions such as differentiation or programmed cell death are difficult to study in pluripotent stem cells, since the consequence of deregulated expression

FIG. 5. Time course of IFN-induced expression in stable ES cell lines. CAT assays were carried out with 25 μ g of protein obtained from MBL-5 ES cell line p1CX 2.1 at 0, 2, 6, 12, 24, or 48 h after treatment with 1,000 IU of IFN per ml. Closed squares, continuous presence of IFN; open squares, removal of IFN from culture medium after 10 h; closed diamonds, removal of IFN from culture medium after 8 h. % Conversion CM; percent conversion of chloramphenicol to acetylated forms.

FIG. 6. IFN induction of RNA transcripts. Cytoplasmic RNA was isolated from four independent stable EFC-1 ES cell transfectants carrying the vector pn6-16DIA grown in the continuous presence of 0 (lanes 1 to 4) or 1,000 (lanes 1+ to 4+) IU of IFN per ml for 24 h. Ten micrograms of RNA was analyzed for the expression of human DIA by Northern hybridization. Lane C, nontransfected parental ES cell RNA.

is terminal alteration of cell identity. The ability to establish stable cell lines in the absence of transgene expression, and subsequently to express heterologous genes in a precisely controlled manner, should help to overcome this difficulty. The extremely low levels of basal expression directed by the 6-16-based expression vectors means that this approach should be applicable to molecules that are biologically active at low concentrations. Inducible expression systems could also be used for the controlled inactivation of stem cell functions by the inducible expression of antisense transcripts. The versatility of the system is extended by the ability to alter expression levels by manipulation of the IFN concentration. This should allow the analysis of factors that are toxic to stem cells when overexpressed and the analysis of factors that can have variable biological activities depending on the level of expression.

Comparison of clonal cell lines under induced and noninduced conditions would allow the developmental response of cells or embryo to be correlated directly with expression of the transgene. The developmental consequences of heterologous gene expression could readily be analyzed in vitro. Moreover, it may be possible to investigate the developmental consequences of transgene expression in vivo, by blastocyst injection following induction in vitro, or by culturing chimeric embryos in the presence of IFN.

The general properties of the 6-16 expression system identify it as an appropriate system for use in ES cells. The availability of these vectors increases the experimental versatility of ES cells and in particular affords new possibilities for the identification and characterization of genes which regulate growth, survival, and differentiation processes in the early mammalian embryo.

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