Epstein-Barr virus episome-based promoter function in human myeloid cells

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ABSTRACT

Epstein-Barr virus (EBV) episomal replicons offer an expeditious means for amplifying transfected genes in human cells. A panel of EBV episomes was constructed to assess the relative utility of five distinct eukaryotic promoter elements for high level and inducible gene expression in stably transfected human myeloid leukemia cells. The Rous sarcoma virus 3' long terminal repeat (LTR) was most highly suited for EBV episome-based gene expression, whereas the lymphopapilloma virus and the SV40 early regulatory elements exhibited substantially lower activities. Chemically responsive promoter elements, such as the SV40 early, human metallothionein II, and rat GRP78 gene promoters, retained their inducibility when EBV episome-based. Differences in gene expression obtained with the episomes reflected differential promoter activity rather than significant variations in episome copy numbers per cell. These observations provide guidelines for the optimal design of EBV episomal expression vectors for human expression work.

INTRODUCTION

Human myeloid leukemia cell lines provide experimental access to hematopoietic cell precursors and their differentiated progeny. These cell lines are useful in a wide array of experimental contexts, such as in studies of hematopoietic differentiation (1, 2), hematopoietic tumor cell biology (3), anti-tumor cell mechanisms (4, 5), and other biological processes (6). Gene transfection strategies offer powerful approaches for dissecting the cellular physiology of human myeloid leukemia cell lines at a molecular level. To date, however, attempts to transfect genes into such cells and attain stable high level gene expression in them have met with only limited success (7-9).

We have been exploring the use of episomal replicons as expression vectors for achieving high level plus-sense and anti-sense RNA transcription in human hematopoietic cells.

Episomal replicons are extrachromosomal, circular DNA structures that replicate autonomously to high copy numbers in eukaryotic nuclei, and in principle, offer an expeditious means for amplifying exogenously introduced genes, while at the same time minimizing positional cis effects resulting from chromosomal integration. Two discrete DNA elements, the Epstein-Barr virus (EBV) origin of replication (oriP) and the EBV nuclear antigen-1 (EBNA-1), together confer an episomal replication capacity to DNA circles in an array of human cell types (10-12). In recent studies, we demonstrated the utility of an episomal vector based upon an EBV motif for effective anti-sense RNA-mediated inhibition of gene expression in a nontransformed human T cell clone (4) and for high level plus-sense cDNA expression in two human myeloid leukemia cell lines (13). The usefulness of EBV episomal replicons for cDNA expression in human B cells (14-16) and non-hematopoietic cells (17) as well as for genomic DNA expression (18) has also been recently established.

In the present report, we describe a comparative functional analysis of a selected panel of constitutive and inducible EBV episome-based eukaryotic promoters in a human myeloid cell background.

MATERIALS AND METHODS

Cell culture

U937 cells (19) were grown in complete medium consisting of RPMI 1640 supplemented with 10% fetal calf serum and L-glutamine (2 mM; M.A. Bioproducts, Walkersville, MD), and gentamicin sulfate (40 μ g/ml) in a 5% CO₂ environment. Culture medium was refreshed every 3-4 days and cells were maintained in a logarithmic phase of growth at all times.

Plasmid constructs

Promoter-chloramphenicol acetyltransferase (CAT) plasmids, in which various promoters are linked to the CAT gene within pBR322 and its derivatives, were obtained from other investigators. p220.2, obtained from Dr. B. Sugden (U. of Wisconsin), is a derivative of p201 (12) in which a short multiple cloning site (BamHI, Xba I, Sal I, Pst I, Hind III) derived from pUC12 has been inserted into the Nar I site of the herpes simplex virus 1 thymidine kinase (HSV-1-tk) gene termination sequence (B. Sugden,

TABLE 1. Promoter-CAT plasmids used in this study

Plasmid	Promoter	Source	Reference
LPV-5'-CAT	LPV 5' LTR	L. Mosthaf	22
RSVCAT	RSV 3' LTR	B. Howard	21
SV2CAT	SV40 early	B. Howard	20
I10CAT	rat GRP78 gene	A. Lee	23
HS1CAT	hMTII _A gene	M. Karin	24
SVOCAT	promoterless	B. Howard	20
TKCAT	HSV-1-tk gene	R. Miksicek	25

personal communication). To assemble promoter-CAT/220.2 plasmids, a two-step protocol was followed, illustrated in Fig. 1 for RSVCAT/220.2. In each case, the first step entailed modification of the promoter-CAT plasmid to permit mobilization of the promoter-CAT cassette by BamHI digestion. Each promoter-CAT plasmid contains a single BamHI site downstream of the CAT gene polyadenylation/termination signal sequence. To create a second BamHI site upstream of the promoter, we blunted (through Klenow fragment fill-in reactions) and inserted phosphorylated BamHI linkers (New England Biolabs, Beverly, MA) at the unique Acc I, Nde I, Sal I, Sma I and Hind III sites of SV2CAT [SV40 early promoter; 20], RSVCAT [Rous sarcoma virus 3' long terminal repeat (RSV 3' LTR); 21], LPV-5'-CAT [lymphopapilloma virus (LPV) enhancer linked in tandem to an enhancerless SV40 early promoter; 22], I10CAT [GRP78 gene promoter; 23], and HS1CAT [human metallothionein II_A (hMTII_A) gene promoter; 24], respectively. For the promoterless SVOCAT plasmid (20), the Hind III site immediately upstream of the CAT gene was converted to a BamHI site. In the second step of each construction, the promoter-CAT cassette was transferred into the unique BamHI site within the multiple cloning site of p220.2. The orientation of promoter-CAT cassettes within promoter-CAT/220.2 plasmids was established by Hind III (SV2CAT, RSVCAT, LPV-5'-CAT) or EcoRI (I10CAT, HS1CAT, SVOCAT) digestions. TKCAT, used as a source of probe for the hybridization studies, was obtained from Dr. R. Miksicek (25).

Transfection procedures

Cells were transfected by either electroporation (26) or protoplast fusion (27) using our previously reported protocols (4).

Derivation of stable transfectants

Hygromycin B (Calbiochem, Inc., La Jolla, CA) was added 72 hrs

post-transfection to U937 cells transfected by either electroporation or protoplast fusion. Preliminary hygromycin B toxicity experiments established that 0.2 mg/ml of this lot of hygromycin B killed the U937 cells by 10-12 days. Cells were aliquoted (5×10^4 cells/ml) into 6-well Falcon culture plates, and hyg^R cells began growing out in 14-16 days. Multiple clones appearing in each independent well were pooled, and analyses were performed approximately 4 weeks post-transfection. The efficiency of stable transfectant derivation was approximately 10^{-5} for electroporation and 10^{-6} for protoplast fusion. CAT assays were performed according to an established method (20). CAT results are expressed as % acetylation = 1-acetylated + 3-acetylated + 1,3-acetylated CPM/total (unacetylated + acetylated) CPM.

Chemical treatment of leukemia cells

TPA (12-0-tetradecanoyl phorbol-13-acetate; Sigma, Inc.) was suspended in 10 mM DMSO at a stock concentration of 1 mg/ml. Inductions were performed on cells maintained at a density of 2×10^5 /ml with TPA at a concentration of 25 ng/ml. After 48 hr, adherent cells (5×10^6) were removed by a rubber policeman and prepared for CAT assays. The calcium ionophore A23187 (Sigma, Inc., St. Louis) was made up in PBS and added to a final concentration of 7 μ M. After 16 hours, the cells were harvested for CAT assays. Cd⁺⁺ was added to a final concentration of 1×10^{-5} M for 16 hr.

Hirt procedure and DNA blots

U937 supernatants containing promoter-CAT/220.2 episomes were prepared according to the method of Hirt (28). DNA aliquots corresponding to 1×10^6 cells were digested with BamHI (4-fold unit excess; N. E. Biolabs), size-fractionated on 0.8% agarose gels, and transferred to Gene Screen (NEN, Boston, MA) by alkaline blotting (29). Probe DNA was labeled by nick translation (BRL, Bethesda, MD) with α^{32} dCTP (NEN, Boston, MA). Hybridization reactions were performed in 50% formamide, 1x Denhardt's solution (0.02% bovine serum albumin/0.02% ficoll/0.02% polyvinylpyrrolidone), 1 M NaCl, 50 mM Tris-HCl, pH 7.6, 2 mM EDTA, pH 8, and 1% SDS at 42°C for 12 hr. Two washes were performed at 68°C with 2x SSC, 1% SDS. After air drying, the filters were exposed to X-OMAT film (Kodak, Rochester, NY) for 48 hr with an intensifying screen. Copy numbers were calculated from densitometric analyses of the autoradiograms performed on a LKB Ultrascan.

Fig. 1 Assembly of RSV-CAT/220.2 episomes. The promoter-CAT cassette (2.1 kb) from RSV-CAT (21) was transferred into the BamHI site of the EBV episomal replicon p220.2 (8.9 kb; see Materials and Methods) in both orientations, α and β . For the RSV-CAT/220.2 schematic shown at the bottom of the figure, the broad bar designates p220.2-derived sequences and the narrow bar indicates pBR322-derived sequences. Shading designations are as follows: solid (right), herpes simplex virus-1 thymidine kinase (HSV-1-tk) gene promoter; solid (left), HSV-1-tk polyadenylation/termination sequence; horizontal cross-hatch, *E. coli* hph gene; dense horizontal cross-hatch, polylinker from pUC12 (SmaI-HaeIII fragment) inserted within the HSV-1-tk polyadenylation/termination sequence; alternating cross-hatch, pBR322 ori; stipple, *amp^r* gene. RSV, RSV 3' LTR; N, Nde; H, Hind III; B, BamHI. Arrows indicate direction of transcription.

RESULTS

EBV episomal replicon-based promoter function

Eukaryotic promoter function within expression vectors can be influenced in *cis* by neighboring sequences and in *trans* by cell-associated or exogenously-introduced regulatory factors. In general, *cis* and *trans* modulatory effects upon promoter activity within expression vectors must be ascertained empirically. The EBV

episome-based activities of a panel of five selected eukaryotic promoters, incorporating their respective enhancer elements, were compared through stable CAT expression experiments. In this experimental system, the prokaryotic CAT gene serves as a reporter for promoter-driven transcriptional activity in eukaryotic cells (30). Promoter-CAT plasmids were modified to permit subcloning of their promoter-CAT cassettes into the EBV episomal replicon p220.2 (Fig. 1; see Materials and Methods). In addition to the EBV sequences required for episomal replication, p220.2 contains the prokaryotic hph (hygromycin-B-phosphotransferase) gene (31) which confers resistance to hygromycin B and serves as a selectable marker. Promoter-CAT/220.2 plasmid constructs assembled in this way incorporated the promoter-CAT (or CAT only) transcriptional cassettes in one of two alternative orientations, designated α and β , with α signifying the orientation in which the promoter element is proximal to the EBV oriP (Fig. 1). The twelve promoter-CAT/220.2 constructs were stably transfected into U937 cells.

CAT enzymatic assays were performed on cellular extracts pre-

Fig. 2 CAT enzymatic activities in stable promoter-CAT α /220.2 U937 transfectants. Each bar represents an independent hyg^R transfectant selected at random for analysis. The I10CAT/220.2 transfectant was derived by protoplast fusion; all other transfectants were generated by electroporation. - and + indicate the absence or presence, respectively, of inducer, as detailed in the text, with - and + bars for independent transfectants sequentially paired.

pared from stable hygromycin B-resistant (hyg^R) transfectant lines derived from multiple independent transfectant wells corresponding to each of the promoter-CAT/220.2 constructs. Among the α -oriented EBV episomal constructs, the one incorporating the RSV 3' LTR consistently yielded the highest levels of CAT expression (Fig. 2). While the α -oriented RSV 3' LTR demonstrated acetylation values ranging from 52-73%, those for the constitutive LPV enhancer and SV40 early promoters were only 6-12% and 0.7-4%, respectively. Similarly, neither of the inducible [hMTII_A gene and glucose-regulated protein 78 Kd (GRP78) gene] promoters exhibited CAT expression levels comparable to the RSV 3' LTR. Similar α -oriented promoter analyses in a second myeloid leukemia cell line, K562, yielded findings that paralleled those for U937, with the RSV 3' LTR exhibiting maximal activity (data not shown). In contrast to the relatively consistent CAT expression levels observed among independent transfectants containing the same α -oriented promoter, considerable variability in expression levels was noted among independent transfectants with the same β -oriented promoter in U937 transfectants (data not shown). Despite this variability, significant α - β orientation-dependent effects could be discerned, with an α preference evident for some promoters (RSV 3' LTR, GRP78 gene) and a β for others (LPV, SV40 early). For both α - and β -oriented promoters, hierarchy of relative episome-based promoter activities remained the same over a one year observation period.

To determine whether inducible promoters function as expected in the context of an EBV episomal replicon, we assessed pre- and post-induction CAT enzymatic activities in transfectants carrying different episome-based inducible promoters. The human metallothionein II_A promoter showed significant and varying levels of basal activity and was inducible in either the α (Fig. 2) or β (data not shown) orientations. Induction ratios for this promoter when α -oriented were 1.2, 1.6 and 6.7 for 3 independent transfectants, and no induction was observed in a fourth. The ionophore-inducible GRP78 gene promoter displayed 2-fold inducibility (Fig. 2). The SV40 early promoter demonstrated striking phorbol ester responsiveness (see below).

Analysis of episomes in Hirt supernatants

In order to ascertain whether differences in CAT enzymatic activity might be a consequence of differences in episome copy

Fig. 3 Analysis of episomes in promoter-CAT α /220.2 U937 transfectants. Hirt supernatants were prepared from four of the U937 cells stably transfected with different promoter-CAT α /220.2 constructs. DNA was BamHI digested, size-fractionated, transferred to Gene Screen, and probed with either (A) a 2.1 kb EBV-oriP fragment or (B) a 0.4 kb fragment of TKCAT (27) corresponding to the 3' end of the HSV-1-tk promoter and the 5' end of the CAT gene. The 8.9 kb bands (A) represent the p220.2 parental vector, and the approximately 2.1-2.2 kb bands (B) represent the excised promoter-CAT cassettes. Lane 1: RSVCAT α /220.2; Lane 2: LPVCAT α /220.2; Lane 3: SV2CAT α /220.2; Lane 4: I10CAT α /220.2.

numbers in transfectants, Hirt supernatants were prepared from representative transfectants with the α -oriented RSVCAT α /220.2, LPV-5'-CAT α /220.2, SV2CAT α /220.2, and I10CAT α /220.2 (uninduced) episomes. CAT activities for these particular transfectants were 73%, 8%, 0.7% and 5%, respectively. Hirt supernatant extra-chromosomal DNAs were digested with BamHI and probed with a 32 P-labeled EBV oriP fragment (Fig. 3a), revealing in each case a prominent 8.9 kb band, corresponding in size to linearized p220.2, as well as faint secondary bands for two of the transfectants

Fig. 4 Phorbol ester inducibility of episome-based SV40 early and RSV 3' LTR promoters. CAT enzymatic activities in U937 cells stably transfected with SV2CAT α /220.2 (lanes 1 and 2) and RSV2CAT α /220.2 (lanes 3 and 4) are shown, comparing 48 hr TPA-induced (lanes 1 and 3) and non-induced (lanes 2 and 4) cells.

(lanes 1 and 4) which could be better visualized with prolonged autoradiographic exposure. Hybridization of these BamHI-digested Hirt supernatant DNAs to a probe spanning the CAT coding region (Fig. 3b) showed bands in the 2.1-2.2 kb size range, corresponding to the sizes of the various promoter-CAT cassettes. Comparison of signal intensities indicated that the episomal gene dosages in these different promoter-CAT α /220.2 transfectants were in a narrow range, and the marked differences in CAT activities seen for them could not be accounted for by the relatively minor variations in episomal copy numbers observed. For instance, the RSV 3' LTR- and LPV-based episomes showed relatively minor differences in episome band intensities, and yet the former yielded a 9-fold higher acetylation value. Semiquantitative densitometric analyses of hybridization intensities, using copy number controls (not shown), indicated plasmid copy numbers ranging from 15-25 copies per cell. The extra 2.0 kb band strongly hybridizing to the CAT probe in the I10CAT α /220.2 transfectant (Fig. 3b) was unexplained but may represent an episome species which has arisen from a deletional event within the I10CAT cassette. Analogous hybridization

experiments performed with genomic DNA in Hirt pellets as target revealed no chromosomally-integrated, EBV oriP-containing vector sequence elements (not shown).

Promoter activity in phorbol-ester-induced U937 cells

U937 cells can be chemically induced to terminally differentiate along the monocytic lineage by phorbol esters (32). To determine whether the function of episome-based promoters is altered in the course of phorbol ester-mediated leukemic cell differentiation, SV2CAT α /220.2 and RSVCAT α /220.2 hyg^R U937 transfectants were induced for 48 hours with TPA (25 ng/ml), differentiation was confirmed using morphologic criteria and cellular extracts were assayed for CAT enzymatic activity (Fig. 4). A marked stimulation of CAT expression in the phorbol ester-treated SV2CAT α /220.2 transfectant was observed, increasing from 15% to 96% acetylation under these enzymatic assay conditions. In contrast, the RSVCAT α /220.2 transfectant displayed a significantly lower (1.5 fold) induction ratio.

DISCUSSION

Our results demonstrate that EBV episomal replicons can be effectively used to stably express exogenous genes in human myeloid leukemia cells. Episomes replicate to high copy numbers in these cells and are maintained in an episomal state on a long-term basis. Furthermore, our promoter analyses have established a hierarchy of EBV episome-based promoter function in stable transfectants, with the RSV 3' LTR proving to be most suitable for constitutively driving high level gene expression in human myeloid cells. This parallels our previously reported finding that the RSV 3' LTR is most optimal amidst the panel studied for episome-based gene expression in human T cell clones (4), and fits nicely with the observation that this promoter, when episome-based, yields high level expression of cell surface proteins in both U937 and K562 myeloid cells (13). Together these observations point to the general usefulness of the RSV 3' LTR for expression work in human hematopoietic cells. In contrast, EBV episomes incorporating the SV40 early promoter yielded significantly lower levels of constitutive gene expression in this system. The LPV enhancer, which is known to be specifically active in a variety of human hematopoietic cell types (22) and has been shown by us to be

nearly as effective as the RSV 3' LTR for constitutive episome-based gene expression in human T cell clones (4), was considerably less active in the myeloid leukemia cell background.

Inducible promoters retain their responsiveness to inducing agents in the context of the EBV episome p220.2, paralleling the behavior of their chromosomally integrated counterparts. The episome-based hMTII_A promoter demonstrated heavy metal inducibility, albeit at a generally lower induction ratio than that achieved with this promoter in other vectors (33). The hMTII_A promoter showed significant basal activity in the absence of inducer, consistent with the presence of a metal-independent enhancer element in this promoter (24). Marked variability in levels of basal activity and inducibility was observed among independent U937 transfectants carrying the hMTII_A episomes, and variability for this promoter in an episomal vector cannot be attributed to integration site effects as for an integrating factor (33). This variability will complicate the use of an episome-based hMTII_A promoter for expression purposes. The rat GRP78 gene promoter similarly displayed its expected ionophore-inducibility and basal activity (23, 34) when episome-based, but again the relative increase in activity observed post-induction was less than that previously reported for this promoter when episome-based in stably transfected human T cells (4) or transiently expressed in human HepG2 hepatoma cells (34).

In contrast to the relatively weak inducibility seen for the hMTII_A and GRP78 gene promoters, the episome-based SV40 early promoter demonstrated striking inducibility in response to its inducer, TPA. A number of cellular (35) and viral (36) promoters are known to be stimulated by phorbol esters, presumably through activation of protein kinase C. The SV40 early promoter contains several TPA responsive elements (37) whose activity are highly dependent upon cell-type (37) and cellular differentiation state (38). This promoter has been shown to be phorbol ester inducible in HepG2 cells (36), and we have here demonstrated that it retains striking inducibility within an EBV episome in U937 myeloid cells. The activity of the RSV 3' LTR, the strongest constitutive promoter in our study, was marginally upregulated by TPA in U937 cells, whereas in HepG2 cells, phorbol ester responsiveness was not observed for it in transient expression studies (36).

The phorbol ester responsiveness of these episome-based

promoters is of particular interest in the context of human myeloid leukemia cells. Such cells serve as experimental models for the study of hematopoietic differentiation and are effectively induced to terminally differentiate with TPA (1, 2, 32). Our findings that both the SV40 early and RSV 3' LTR promoters respond positively to TPA in U937 cells establish the utility of these promoters for transfection studies of phorbol ester-induced human myeloid leukemia cell differentiation.

Promoter function within episomal replicons could in principle be modulated in cis and/or in trans. The EBV oriP has been reported to exert cell-specific cis transcriptional enhancer effects upon the SV40 early (enhancerless variant) and HSV-1-tk promoters (16). Orientation-dependent cis effects upon promoter function within p220.2 were observed but did not fit a predictable pattern. Whereas the α orientation (EBV oriP proximal) was more optimal for the RSV 3' LTR and GRP78 gene promoters, the remaining promoters, including the SV40 early promoter, showed a β orientation (EBV oriP distal) preference. Our findings cannot be readily reconciled with those of Young et al. (17) who showed that the α orientation is the preferred one within p220.2 for several enhancer-promoter combinations, including the cytomegalovirus enhancer-SV40 early promoter, for expression in CV-1 monkey kidney cells and human embryonic 293 kidney cells. It is possible that this discrepancy reflects cell-, promoter-, or gene-specific differences.

A detailed comparison of the relative activity of each promoter in episomal and chromosomal contexts was beyond the scope of the present study. Such comparative analyses of promoter function are complicated by gene dosage considerations, since for integrating vectors, multiple gene copies can integrate at multiple chromosomal sites (39), where cis effects between neighboring sequences and among tandem integrants further obscure the picture.

Exchanging promoter elements within the p220.2 EBV episomal replicon did not affect episomal replication capacity in a dramatic fashion, implying that the particular promoter elements chosen for study here do not exert marked cis effects upon EBV oriP's replicative function. The minor variations in episome copy numbers revealed by our Hirt hybridization analyses could not account for the highly divergent CAT enzymatic activities observed in different transfectants, and this leads us to conclude that the variations in CAT activities observed for different episome-based

promoters are indeed explained by differential promoter activity and not by variability in episomal amplification. Furthermore, our episomal hybridization studies demonstrated the presence of aberrant bands in 2 of 4 transfectants examined, probably reflecting early post-transfection mutagenic events rather than ongoing episomal instability (14, 40).

In summary, we have established the hierarchy of EBV episome-based activities for a selected panel of constitutive and inducible eukaryotic promoters in a human myeloid cell line. The promoter-CAT/220.2 episomes that were constructed for this study will be useful for comparative analyses of EBV episome-based promoter function in other human cell types. In addition, we have shown that these promoters are subject to their normal regulatory constraints in their amplified state and extrachromosomal location. These experimental data provide important guidelines for the choice of promoters in the assembly of EBV episomal expression vectors suitable for transfection work in human cells.

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