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Interference of mouse GATA-1 function by the glucocorticoid receptor at the level of the beta-globin gene and influence of GATA-1 on Friend leukemia virus (FLV) genome expression: Relationships to the inhibition of mouse erythroleukemia cell differentiation by glucocorticoids and to FLV erythrotropism

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City University of New York, 1993

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INTERFERENCE OF MOUSE GATA-1 FUNCTION BY THE GLUCOCORTICOID RECEPTOR AT THE LEVEL OF THE BETA-GLOBIN GENE AND INFLUENCE OF GATA-1 ON FRIEND LEUKEMIA VIRUS (FLV) GENOME EXPRESSION: RELATIONSHIPS TO THE INHIBITION OF MOUSE ERYTHROLEUKEMIA CELL DIFFERENTIATION BY GLUCOCORTICOIDS AND TO FLV ERYTHROTROPISM.

by

TAI-JAY CHANG

A dissertion submitted to the Graduate Faculty in Biomedical Science in partial fulfillment of the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

INTERFERENCE OF MOUSE GATA-1 FUNCTION BY THE GLUCOCORTICOID RECEPTOR AT THE LEVEL OF THE BETA-GLOBIN GENE AND INFLUENCE OF GATA-1 ON FRIEND LEUKEMIA VIRUS (FLV) GENOME EXPRESSION: RELATIONSHIPS TO THE INHIBITION OF MEL CELL DIFFERENTIATION BY GLUCOCORTICOID AND TO FLV ERYTHROTROPISM.

by

Tai-Jay Chang

Adviser: Dr. William Scher

Treatment of Friend leukemia virus (FLV)-infected mouse erythroleukemia (MEL) cells with hexamethylene bisacetamide (HMBA) induces a program of erythrodifferentiation as judged by an increase in the synthesis of globins and other erythroid-specific products. This induction can be inhibited by glucocorticoids, *e.g.*, dexamethasone (DEX). All globin and other erythroid-specific genes tested contain GATA-response elements (GATA-RE) and can be transactivated by GATA-1, a major erythroid transcription factor. GATA-1 is highly expressed in erythroid cells, including MEL cells. A glucocorticoid receptor (GR) response element motif was noted near a GATA-RE motif in the promoter region of the mouse α_1-globin, ß-major and ß-minor globin genes and in the FLV long terminal repeat (LTR) and, therefore, the possibility that the DEX-inhibition of induced MEL cell differentiation may involve effects of the GR on GATA-1 activity was investigated. Evidence obtained from transfection assays and DNA electrophoretic mobility shift assays indicates that the GR binds GATA-1 and interferes with its function prior to any interaction with DNA, and that the presence of a GRE near a GATA-RE augments the GR effect. The N-terminal 106-amino acid-domain of the GR was found to be essential for the effect possibly by binding to GATA-1. Since GATA-1 is autoregulatory, *i.e.*, it has been shown by others to bind to its own promoter and upregulate its own transcription, the finding that activated GR can interfere with GATA-1

function may provide an explanation for the inhibition by glucocorticoids of the entire program of erythroid differentiation in MEL cells. That is, by interfering with GATA-1 function, the GR can not only inhibit the expression of erythroid structural genes, but also the expression of a regulatory gene, GATA-1 itself. In addition, it was shown that a GATA-RE in each of the ß-globin promoters and in the FLV LTR responds to mouse GATA-1 in a functional transfection assay. FLV can infect many, if not all, cell types, but causes pathogenic effects primarily in cells of the erythroid lineage, and therefore is considered erythrotropic. The LTR response to GATA-1 appears to at least partially explain the mechanism of the erythrotropism of FLV.

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Introduction

The investigational goals of this thesis were to understand the mechanism(s) of the inhibition of induced MEL cell differentiation and retroviral reverse transcriptase activity due to glucocorticoids. The plan was to determine if molecular interactions affected response element function in erythroid genes, and if so, if these interactions occurred between transcription factors. The transcription factors studied were a major erythroid factor, GATA-1, and a member of the nuclear superfamily of ligand-binding transcription factors, the glucocorticoid receptor (GR). The possibility of such interactions stems from studies that demonstrated inhibition by glucocorticoids of induced erythroid differentiation and reverse transcriptase activity in mouse erythroleukemia (MEL) cells (1-3). Of these glucocorticoids, dexamethasone (DEX), has generally been used in subsequent studies (4). Studies of gene regulation at the present time generally involve aspects of transcription factors (TFs) such as their level, location (nucleus), activity (functional form(s)), and response elements (REs) (5), higher orders of DNA structure (6, 7), and/or altered primary structure of DNA, e.g., methylated cytosine (8-11).

In considering the inhibitory effect of DEX on MEL cell differentiation and inspecting mouse globin promoters and the Friend leukemia virus (FLV) long terminal repeat (LTR), a putative glucocorticoid response element (GRE) consensus sequence with a "perfectly conserved" primary hexamer was noted to be present only 7 bases from a GATA response element (GATA-RE) known to bind GATA-1 centered about 207 bases 5' of the ß-major (ß-maj) globin cap-site, another putative GRE was noted to actually overlap a putative GATA-RE which is centered about 204 bases 5' of the ß-minor (ß-min) globin cap-site, and a putative GRE was noted to overlap a putative GATA-RE at approximately 180 bases 5' of the cap-site of FLV LTR (Fig. 1) (4). Therefore, it was thought that DEX-activated GR might interfere with GATA-1 stimulation of mouse ß-globin and FLV transcription and that this inhibition might be important in the DEX-mediated inhibition of MEL cell differentiation and MEL cell retroviral activity.

Transcriptional control of hematopoietic cell differentiation: All mature hematopoietic cells, including erythrocytes, megakaryocytes, granulocytes, monocytes, and lymphocytes, are derived from pluripotent stem cells located in the bone marrow of the adult mammal (12). As these stem cells proliferate, they become committed to individual hematopoietic lineages. Lineage-specific transcription factors should play a key role in determining the developmental fate of precursor cells (13). The recent characterization of

cis-acting DNA elements in lineage-specific genes has allowed the subsequent isolation of transcription factors that bind to these regulatory sites. To understand how red blood cell differentiation is programmed, erythroid transcription factors have been identified on the premise that either these proteins or their regulators specify the fate of erythroid progenitor cell. One erythroid transcriptional factor, GATA-1, (14) is discussed below.

Globins: Mouse globins are expressed from two loci: one on chromosome 11 contains the sequences of three α–type globins, 5'-embryonic ζ, adult α_1, adult α_2-3', and another on chromosome 7 that in the mice with the Hbb^d haplotype, studied here, contains the sequences of 4 coding and 3 pseudo ß-type globins: 5'-embryonic Y, pseudo bhO, embryonic Z, pseudo bh2, pseudo bh3, adult ß-maj and adult/fetal-like ß-min-3'. "The globin gene families have long served as paradigms for the study of developmental regulation of gene expression" (5). Interest in the regulation of these genes has been fueled by many factors including their tissue-specific (erythroid) and exceedingly high level of expression, their inducibility in *in vitro*-cultured cell lines, the availability of naturally occurring mutants specifying altered globin gene regulation such as those for the hereditary persistence of fetal hemoglobin (Hb) (15), and the desire to devise treatment for ß-hemoglobinopathies: sickle cell anemia and ß-thalassemias (14, 16, 17). One therapeutic goal being sought for ß-hemoglobinopathies is the up-regulation of normal fetal ß-globin since it is known that subjects that display both hereditary persistence of fetal Hb and ß-thaleassemia have ameliorated symptoms compared to those that occur with the thalassemia alone. Several conditions have been found to increase the level of fetal Hb in relation to adult Hb in *in vivo* and/or primate tissue culture systems: erythropoietic stress due to hypoxia/anemia which, if acute, increases the levels of erythropoietin, a physiological erythropoietic agent, and treatment with several agents (5-azacytidine, 5,6-dihydro 5-azacytidine, arabinosylcyctosine, and hydroxyurea which interfere with DNA synthesis, vinblastine, a mitosis inhibitor, and butyrate and butyrate analogs) (17-19). Although mice have no exact counterpart of primate fetal globin (only embryonic and adult ß-type globins), the adult mouse ß-min globin has some fetal characteristics (20) and its synthesis can be up-regulated in comparsion to that of the primary adult mouse ß-globin, ß-maj globin. Treatments that were shown to increase the production of erythroid cells, such as erythropoietin adminstration to sheep (21) or the induction of (erythropoietic stress) anemia in mice (22) were associated with elevated levels of fetal or fetal-like ß-globins. Erythropoietic stress induced mouse ß-min globin is not due to a decrease in the degradation of the adult ß-globin or its mRNA and therefore is controlled at a

pretranslational level since an increase in the synthesis as well as the level of ß-min compared to that ß-maj globin was observed (22).

Induced MEL cell differentiation: MEL cells have been used as a model system for studying differentiation in general, erythroid differentiation in particular, the regulation of differentiation (erythroid)-specific gene expression, the relationship of cell differentiation to maligancy, and the effects of a retrovirus on differentiation and malignancy (23-25). MEL cells were derived from tumors that were initiated with injections of spleen cell suspensions prepared from FLV-infected mice at a "late" time after infection. At this time these cells are "transformed" and can grow as long-term lines and can induce solid tumors when "reinjected" subcutaneously into syngeneic mice (26, 27). They were the first cell lines developed for the study of globin gene expression. Over 300 agents have been shown to induced MEL cells to undergo erythrodifferentiation (including production of α-, ß-maj and ß-min globins and "complete" Hbs) and several, although not all, of these agents induce commitment to terminal cell division with loss of maligant potential (28). The inverse relationship between differentiation induction and loss of malignant potential in these cells appears to be the first experimental evidence for all of subsequent the studies aimed at the development of "differentiation chemotherapy" (23). The inducers can be placed in groups depending upon 1) the relative levels of ß-maj and ß-min globins they induce which appear to reflect primarily the levels of induction of the ß-maj and ß-min globin mRNA expression (29-31), 2) their sensitivity to glucocorticoid inhibition of their differentiation inducing activity, 3) their ability to induce commitment to terminal cell division (32), and 4) their ability to induce differentiation in certain differentiation-resistant variant MEL cell lines (33, 34). For example, the most studied of these compounds, dimethyl sulfoxide (DMSO), hexamethylene bisacetamide (HMBA), butyrate, and hemin, can each be placed in a different group due to the following four findings. 1) Approximately 70% of the ß-globin induced by DMSO and HMBA consists of ß-maj, butyrate generally induces 51% ß-maj (and in one cell line, embryonic ß-like globin) (35), and hemin induces only about 20% ß-maj (36). 2) Induction due to DMSO, HMBA, or hemin is inhibited by glucocorticoids while that due to butyrate is not (24, 37). 3) Whereas DMSO, HMBA, and butyrate all can commit MEL cell to terminal cell division, hemin cannot (37). 4) Lastly, a variant line, DR-10, was developed that is highly induced in response to HMBA, but not to DMSO (33).

Inhibition of MEL cell induced differentiation: In addition to studies of MEL cells with different inducing agents, there have been many studies of agents that

inhibit induction of MEL cell differentiation (24, 38). Of these, glucocorticoids (particularly DEX) have been among the most studied. As indicated above, DEX substantially inhibits induction due to DMSO and HMBA, but only modestly that due to hemin, and doesn't inhibit induction by butyrate or its analogs (24, 37). Many of the effects that steroids cause are due to effects on transcription (39) following interactions with steroid-binding receptor TFs (40). The effect of DEX on MEL cell erythrodifferentiation is thought to be due, at least partially, to transcriptional effects (41). DEX binds to a TF (glucocorticoid receptor, GR) which then acts at specific GREs to regulate transcription as discussed below. Further, the effect of DEX on induction was then hypothesized to be due to interactions of erythroid-acting TFs and activated GR.

Response elements in globin genes: Work in several laboratories, suggests that the basis for the various effects of the inducers could lie, at least partially, in differences in their abilities to influence different REs in erythroid-specific genes, e.g., (42, 43) as well as in other genes (44, 45). Since the activities of these REs are generally thought to be due to interaction with TFs, the inducers may affect the level and/or activity of TFs. Several sequence motifs near one or more globin genes have been found to be required for TF binding and/or transcritional regulation (5, 14, 17, 46). These TF binding sites (REs) include TATA, CCAAT, CACCC (an Sp-1-like binding site), GATA, and AAGCCAGTG sequences, an NF-E2/AP-1-like (TGACTCA) binding site, another Sp-1 like binding site (poly-G sequence of 16 G's), a 10 base directly repeated sequence (ßDRE), and an octamer binding site, ATTTGCAT. The mouse ß-maj globin gene contains several of these sequences in its promoter region: TATA, ßDRE, CCAAT, CACCC, GATA, AAGCCAGTG. All of these may function in ß-maj transcription, but no such sites have been characterized, as yet, in the ß-min globin gene. In addition, several of these motifs are also present, bind TFs, and function in globin transcription in at least four other globin gene regions: 1) the locus control region (LCR), 2) a coding sequence, 3) an intervening sequence, and 4) the 3'-enhancer region. Four of these motifs (ßDRE, AAGCCAGTG, octamer, and NF-E2) in a chick, human, or mouse globin gene have been shown to respond to one or more of the inducers discussed above (35, 42, 47-50). These inducer-responsive motifs have so far been found in the promoter or just 5' to it and in the LCR. Although there are overlapping specificities in the "inducer-specific"-responsive REs, the REs regulating the ß-globin complex can tentatively be classified in regard to their inducer-responsiveness into five groups: 1) erythroid/megakarocyte/mast-specific such as GATA, 2) adult erythroid stage-specific such as the AAGCCAGT consensus sequence which responds to DMSO to stimulate adult ß-globin transcription (46, 51, 52) and the

ßDRE which also responds to DMSO (53) as well as HMBA (54), 3) fetal or fetal-like erythroid stage-specific REs such as the NF-E2/AP-1 site which responds to hemin, 4) embryonic erythroid stage-specific REs which respond to butyrate (42), and 5) REs such as TATA, CCAAT, and CACCC which respond to ubiquitous factors and are not erythroid-specific.

GATA-1: Of these motifs, the GATA-RE has been found to be active in all globin genes tested (including mouse α1- and ß-maj globin genes) and at least 8 other erythroid-specific, but non-globin, genes (55). The GATA-RE responds to the erythrocyte/megakaryocyte/mast cell-specific TF, GATA-1 (56), is essential for normal erythroid development (57), and is present at high levels in erythroid (including MEL) cells. The regulation of GATA-1 levels appears to be important in its function since its mRNA level is increased during mouse development (58) and somewhat during MEL cell differentiation (59).

The zinc-finger protein GATA-1 is an example of a lineage-specific transcription factor (14). GATA-1 binds to the GATA consensus sequence (A/G)(T/A)GATA(A/G)(G/T) in regulatory regions of the α– and ß-globin gene loci (5, 60, 61) and of other red cell genes (62-64). GATA-1 is first expressed at low levels in multipotential hematopoietic progenitor cells, up-regulated during erythroid maturation (55, 65, 66), and down-regulated during myeloid differentiation (66). The expression of GATA-1 is subsequently restricted to three different hematopoietic lineages: erythrocyte, megakaryocyte, and mast cell (5, 59, 67, 68). GATA binding sites are cis-acting and play an important role in the transcription of globin and non-globin genes in red cells. Mutagenesis of GATA sites in these genes results in lower levels of their transcription. Mutation of GATA motifs in the promoters of genes expressed in megakaryocytes and mast cells yields the same results indicating that GATA sites also are important for transcriptional activity in these lineages as well (56, 68). GATA-binding sites are also present in the active domain of globin locus control regions, i.e., DNase I-hypersensitive segments required for activation of the entire chromatin domain and for high-level, position-independent expression of α–globin (43, 69, 70) and ß-globin (71-73) genes.

GATA transcription factors: GATA-1 is a member of a multigene family (73). Four distinct members of this family have been described in vertebrates thus far: GATA-1, GATA-2, GATA-3, and GATA-4 (56, 74-76). Members of the GATA-binding protein family are related by virtue of a highly conserved protein domain that is necessary and

sufficient for DNA-recognition (67). These proteins are related by homologous zinc-finger domains with the configuration Cys-X_2-Cys-X_{17}-Cys-X_2-Cys. Members of the GATA-binding protein family differ in their tissue distribution. Whereas GATA-1 mRNA has been found only in the three hematopoietic lineages mentioned previously, GATA-2 is expressed in large number of different cell types, including endothelial cells (56, 75). GATA-3 is also found in a variety of cells, including T lymphocytes, and serves as a transcription factor for several genes expressed only in T cells, such as the subunits of T cell antigen receptors (77). GATA-4 is expressed in heart, intestinal epithelium, primitive endoderm, and gonads (76).

GATA-2 and GATA-3 are expressed in addition to GATA-1 in erythroid cells at specific developmental stages (74). All four family members bind in vitro to the same consensus sequence with high affinity and stimulate transcription from reporter constructs in heterologous cells (74, 78). Transcription factors are modular proteins (79). In general, DNA-binding domain(s) are separate from regions that mediate interactions with other proteins. Among the GATA-proteins, GATA-1 and GATA-4 are potent transcriptional activators, whereas GATA-2 and GATA-3 are more modest (55). In mouse GATA-1, qualitatively different domains have been been identified by fusion of N- and C- terminal regions to a heterologous DNA-binding domain (67). The N-terminal domain confers transcriptional activation in this assay, whereas the C-terminal portion does not. Existing evidence indicates that GATA-1 plays an important role in regulation of the erythropoietin (Epo) receptor (EpoR) promoter; however, it cannot be the sole factor responsible for its expression (80, 81). Nonetheless, involement of GATA-1 in EpoR expression is required for viability and subsequent development of erythroid progenitors. GATA-1 is autoregulatory (82). It binds to a double GATA motif in its own promoter and upregulates its own transcription.

Steroid hormone receptors: Steroid hormones exert many effects on their target cells including effects on cell growth, differentiation and the level of specific proteins. Steroid hormones exert these actions after binding to specific receptors which are localized primarily within the nucleus. All of these receptors have the same general structure as well as a high degree of homology in their hormone-binding and DNA-binding regions. The glucocorticoid receptor (GR), 94 KDa, like other members of the steroid receptor family (83, 84), consists of three structural domains, the N-terminal domain, the central DNA-binding domain, and the C-terminal hormone-binding domain. The N-terminal 50 KDa of the receptor is essential for full transcriptional activity. The DNA-

binding domian, comprising approximately 75 amino acids, contains two zinc fingers that confer DNA binding specificity on the receptor. It also contains a basic region which is involved in nuclear localization and possibly in interactions with DNA (85). The hormone-binding domain of approximately 30 KDa, upon binding hormone, promotes nuclear localization, DNA binding, and transcriptional activation. In the absence of hormone, this domain is involved in suppressing receptor activity since mutants lacking this domain are constitutively active. This suppression may be due to interaction with masking proteins such as Hsp90, which forms a complex with the receptor only in the absence of hormone.

The N-terminal domain of the GR is required for full transcriptional activity (86-88) and, since it is not reqired for hormone binding (89), receptor activation (90), dimerization (91) or DNA recognition (92), it has been termed a modulatory domain. Among steroid receptors, the N-terminal domain is the most variable in size and amino acid composition (83), and so it is thought that this domain may be responsible for the receptor's ability to interact with a specific subset of transcription factors and lead to different activities. The N-terminal domain of the GR contains at least one sequence that acts as a transcriptional activator. Since a highly acidic region located between amino acids 196 and 293 of the mGR has been shown to be required for near normal receptor activity (86) and since acidic regions can act as transcriptional activators (93, 94), it is tempting to suggest that this acidic region in the GR plays a role in transcriptional activation. However, removal of the acidic region increases nonspecific DNA-binding properties of the receptor with the result that the difference between specific and non-specific DNA binding in vitro is reduced (95). It has been suggested that the increased non-specific DNA-binding properties of N-terminally truncated receptors is due to their increased positive charge (86). Eriksson and Wrange (91) have presented evidence that loss of the N-terminal domain of the GR results in altered protein-protein contacts in GR dimers and in altered protein-DNA interactions.

The DNA-binding domain contains two fingers formed by the tetrahedral co-ordination of two zinc atoms by four pairs of cysteines (92). α-Helices are located immediately adjacent to the distal side of each finger (Ser_{447}-Glu_{457}, and Pro_{481}-Gly_{492}), and are orientated perpendicular to each other. There is a type 1 and a type 2 turn near the beginning of the second finger (Arg_{467}-Cys_{470}, and Leu_{463}-Gly_{466}). In addition there is a small region of an antiparallel ß-sheet involving amino acids Cys_{428} and Leu $_{429}$ and Leu_{463}-Gly_{466}, in the first finger. Härd (96) has developed a model of GR binding to DNA in which one GR binds to each half of the GRE palindrome. The so-called recognition α-helix of each receptor binds in the major groove and interacts directly with GRE-specific

bases. The two halves of the receptor dimer interact directly in the region of the first pair of cysteines of the second finger. Some of the amino acids in the first zinc finger of the GR are involved in determiningthe specificity of DNA binding (97). The amino acids of the second finger that appear important for protein-protein interaction face away from the DNA so that they could conceivably interact with non-DNA components of the transcriptional machinery. Adjacent to the second zinc finger is a region enriched in basic amino acids. A basic region is present in all steroid receptors at approximately the equivalent position although the actual sequence is not conserved. It has been suggested that this region is important for nuclear localiztion of the GR since its attachment to other proteins confers nuclear localiztion (85).

The C-terminal domain of the GR encompasses approximately 262 amino acids and is required to form the hormone-binding site. This domain also contains the binding site for Hsp90 and possibly for other receptor-associated proteins. As noted above, receptor lacking the C-terminal hormone-binding domain constitutively activates transcription, suggesting that in the intact receptor this domain represses activity in the absence of hormone. This repression is possibly due to the interaction with Hsp90 since it is relieved by hormone binding. In addition, the hormone-binding domain also contains a hormone-dependent transactivation domain (98) and a nuclear localization sequence noted above (85), both of which are activated upon hormone binding.

Erythrotropic viruses: Viruses frequently promote pathological effects primarily in a single, or a limited number of, cell type(s). The tissue-specificity of a virus may depend upon its ability to enter cells, for example via a specific receptor that is only present on a particular cell type(s), to the ability of the virus to replicate in a particular cell, or to a combination of these mechanisms. Only a few viruses are known to be tropic for the erythroid lineage, e.g., parvovirus B19 (99-101) and certain retroviruses: avian erythroblastosis virus (15), feline leukemia virus subgroup C (102) and the mouse Friend (103) and Rauscher (104) leukemia viruses.

Friend leukemia virus: FLV can enter several cell types (103, 105-107) apparently due to an evidentally ubiquitous plasma membrane receptor (108). However, the known pathologic effects induced by FLV appear to be essentially limited to erythroid and, to a lesser extent, megakarocytoid tissue, i.e. erythroleukemia and thrombocytopenia can be induced (103) and cells transformed by FLV infection exhibit erythroid features (109) and a megakaryocytoid feature (110). Two domains of the FLV genome are required

for the virus' pathological effects: the regions coding for the env gene and for the LTR (111-113). Recently the mechanism for the erythrotropism of the env gene has been elucidated by the demonstration that the product of this gene, gp55, can mimic the growth-promoting effects of Epo by binding to and activating the EpoR (114). The general mechanism of the LTR has also been explained, but not its relationship to the erythrotropism of the virus. That is, the LTR contributes to FLV pathogenicity following integration of the viral genome near a gene of the ets family providing a DNA enhancer element(s) that is capable of up-regulating the transcription of that gene. For a recent discussion of this mechanism see (115). Although a specific region of the LTR has been shown to contain an element that appears to be responsible for this up-regulation (116), neither the specific element(s), nor the transcription factor(s) that binds to this element are known. In considering which characteristics FLV target (erythroid and megakaryocytoid) cells have in common that might account for the virus' dualtropism and involve the LTR, the transcription factors GATA-1 and SCL appeared to be possible candidates (55, 61, 68, 117) since both appear to be required for erythroid maturation, are expressed in erythroid and megakaryocytic cell lineages, and are down regulated during myeloid differentiation (57, 58, 66, 117, 118). GATA-1 is also down-regulated in megakaryocytic differentiation (119). However, of the two factors, GATA-1 seemed a more likely candidate, since it is not known to be expressed in any granulocytic/monocytic cell lines whereas SCL is (117). Therefore, we searched the identified LTR region for the presence of a GATA-RE motif (120). Such a sequence was found approximately 180 bases 5' of the cap-site.

In considering possible mechanisms for DEX-mediated inhibition of MEL cell differentiation, the sequences of mouse globin genes were searched for GREs. GRE motifs were found in the promoter regions of all three globin genes examined: at -48, -222, and -202 base pairs (bp) 5' of the cap-site of the α_1-, ß-major, and ß-minor globin genes, respectively (Fig. 1) (121, 122). Moreover, these motifs were located near response element (RE) motifs for the major erythroid DNA-binding protein, GATA-1 (7, 14, 59). The GATA-RE consensus consists of an octamer with a generally invariant central GATA seqence flanked by variable dinucleotides (A/G A/T GAT A/T A/G G/T). GATA-1 is present at high levels in MEL cells and is thought to play an important role in erythrodifferentiation (67, 123). Therefore, the observed juxtapositions of response elements for these two transcription factors in erythroid gene promoters raised the possibility that DEX might inhibit MEL cell erythrodifferentiation by activating GRs which by acting at these GREs would interfere with the function of a neighboring GATA-1/GATA-RE complex.

The regions of the α- and ß-globin promoters approximately 200 bp from their respective cap-sites were chosen for initial studies. The general structures of the promoters of all three of these globin genes are similar, with TATA boxes located approximately 30 bp 5' of, and GATA motifs approximately 200 bp 5' of, each cap-site (Fig. 1). It has been shown that the GATA-RE located 184 bp upstream from the α_1-globin gene cap-site, binds to, and can be activated by mouse GATA-1 (mGATA-1) (67) and that the similarly located GATA-RE in the ß-maj globin gene (209 bp upstream from the cap-site) can also bind mGATA-1 (62). In addition, FLV presumably responds to another transcription factor, the GR, since it has been shown that glucocorticoid treatment of FLV-infected cells increases the release of viral reverse transcriptase activity from the cells (1). However, although putative GREs have been noted in the FLV LTR (124), to my knowledge, transactivation of the LTR by the GR has not been demonstrated.

It was demonstrated here that the GATA-RE motifs located approximately 200 bp 5' of the cap-site of each of the mouse ß-globin genes and of the FLV LTR can be activated by GATA-1 and that GATA-1 stimulation of α- and ß-globin and FLV promoter activity is inhibited by "DEX-activated" GRs. This inhibition appears to depend primarily on direct and/or indirect interactions between the GR and GATA-1 before they bind to their respective REs with the presence of a GRE nearby a GATA-RE augmenting the inhibition.

RESULTS

Effect of DEX and charcoal-treated FBS on induced MEL cell differentiation

MEL cells were tested for the response to DEX in the presence of untreated or charcoal-treated (steroid-reduced) FBS (Fig. 2). As expected, cultures incubated in the absence of HMBA either with or without DEX did not produce significant levels of benzidine-staining (hemoglobin-containing) cells regardless of the type of FBS utilized (lanes 1 and 3 of Groups 1-3). In the presence of untreated FBS, HMBA induced 60% of the cells to produce hemoglobin (Group 1, lane 2) and concomitant DEX treatment reduced this level by half (Group 1, lane 4). In cultures grown in the presence of steroid-reduced FBS (Group 2), HMBA induction was more potent, yielding cultures that contained 90% benzidine-stained cells (lane 2) suggesting that steroids present in untreated FBS may partially inhibit HMBA induction. DEX treatment inhibited this induction 43% (lane 4). In

attempting to increase the DEX effect, cultures were pretreated for 1 day with DEX and then subdivided and grown for 4 more days either in the presence or in the absence of HMBA and/or DEX (Group 3). DEX pretreatment inhibited HMBA induction (*cf.* lane 2 in Group 2) and in Group 3). The persistence of the DEX effect after its removal from the medium was presumably due to significant levels of DEX and/ or active GR remaining in the cell after the removal of extracellular DEX. DEX pretreatment plus continued DEX treatment yielded, as expected, a greater inhibition, *i.e.*, approximately 78% (*cf.* lane 4 of Groups 2 and 3). In summary, apparently glucocorticoids present in untreated FBS reduced the level of induction by HMBA, charcoal treatment of FBS increased the sensitivity of the cells to exogenous DEX, and pretreatment with DEX increased its effect.

Effects of mGATA-1 on globin gene promoters and FLV LTR transactivation in CV-1 cells

In order to determine if the inhibition of induced MEL cell differentiation by DEX might be related to interference of GATA-1 function at GATA-REs in the promoters of the mouse globin and FLV genes by "DEX-activated" mouse glucocorticoid receptor (mGR), preliminary studies of the regulation by mGATA-1 of these promoters in cells that do not constitutively produce mGATA-1 were performed. Then effects on GATA-1 regulation by a transfected mGR-expressing plasmid in the presence and absence of DEX were tested. Green African simian kidney CV-1 and/or COS cells have been used as test systems for transcriptional regulation by transfected GATA-1 (67) and the GR (125) since these proteins are either not constitutively expressed or expressed only at very low levels in these cell lines. Transfected reporter plasmids driven by promoter sequences of the mouse α_1-, ß-major, and ß-minor globin genes have been shown to be transcriptionally active in monkey kidney cells (67, 126), but of these promoters, only that of the α_1-globin promoter has been tested and demonstrated to respond to a transfected mGATA-1-expressing plasmid (67). The GATA sequence in the ß-maj globin promoter, although evidentally not tested for response to GATA-1, has been shown to bind it (62). Although the FLV LTR has been shown to bind several factors in nonerythroid cell extracts (127), GATA-RE binding was not noted presumably because no GATA-1 present. In addition, an FLV LTR fragment was tested in EMSAs with MEL cell extracts, but since the fragment tested did not contain a GATA sequence, no GATA-1 binding was noted in this study either (128). As noted above, a MEL cell factor(s) was shown to bind the FLV LTR, but neither the response element(s) or its cognate TF was identified (116).

Increasing amounts of a plasmid expressing functional mGATA-1 were cotransfected with the same amount of a CAT-reporter plasmid linked to the mouse ß-min, ß-maj, α_1-globin and FLV promoter regions containing the GATA-RE and GRE motifs discussed above (Fig. 1). CAT expression driven by all four promoters was increased in an mGATA-1 concentration-dependent fashion (Fig. 3A, C). The ß-maj globin promoter responded to mGATA-1 with greater sensitivity than the ß-min promoter which, in turn, was more sensitive than the α_1-globin promoter. The α_1-globin construct utilized, containing the GRE motif, was previously not found to be efficiently transactivated in COS cells by GATA-1, but a shorter (20-mer) construct containing the GATA-RE, but lacking the GRE motif was (67). However, it was observed here, in CV-1 cells, that both of these reporter plasmids were GATA-1-responsive, (Fig. 3 A, B). These findings demonstrate (1) that the GATA-RE in the ß-maj globin promoter can be transactivated by GATA-1, (2) the similarly located GATA-RE in the ß-min globin promoter is also mGATA-1-responsive, (3) that a reporter plasmid containing either a "full-length", or a 20-mer sequence of the, α_1-globin promoter can be transactivated by mGATA-1 (Fig. 3A, B). (4) that the GATA-RE in the FLV LTR also can be transactivated by GATA-1. The third finding is of particular interest because in both COS and 3T3 cells, only the 20-mer, but not a "full-length", α_1-globin promoter sequence was found to be responsive to mGATA-1 (67). The lack of response of the "full-length" promoter in that study appears to have been related to the difference in cell types used. The fourth finding appears to explain the reason that the LTR is required for the erythrotropism of FLV.

Activated GR inhibits mGATA-1 transactivation

Cotransfections were performed with the same amount of the mGATA-1- and mGR-expressing plasmids along with four different globin- and the FLV promoter-driven reporter plasmids in the presence of various concentrations of DEX (Fig. 4). In the presence of the mGR plasmid, DEX treatment inhibited mGATA-1-directed transcriptional stimulation of all reporter plasmids tested in a concentration-dependent manner. It appeared that the GRE was not required for the "DEX-activated" GR down-regulation of mGATA-1 function, since the α_1-globin reporter plasmid containing the 20-mer sequence that lacked a GRE motif was down-regulated 63% by "DEX-activated" GR (Fig. 4B). However, the GRE motif did appear to contribute to the effectiveness of the GR, since, at least at the highest concentration of DEX tested (1 µM), the longer α_1-globin promoter construct that contained a GRE motif yielded a greater "DEX effect", *i.e.*, 85 % down-regulation (Fig. 4A). Neither the mGR-expressing plasmid in the absence of DEX, nor DEX in the absence

of the mGR-expressing plasmid significantly down-regulated the GATA-1 effect (not shown).

Binding of MEL cell nuclear factors to GATA-RE and GRE motifs in mouse globin promoters

The alteration of mGATA-1 function due to "DEX-activation" of the GR suggested that the inhibition of induced MEL cell differentiation by glucocorticoids may be related to effects on mGATA-1 function and/or cellular concentration. MEL cells have been shown to contain GRs (3, 129-133), mGATA-1 transacting activity (67, 123), and GATA-1 DNA-binding activity to mouse α_1- and ß-maj globin sequences has been demonstrated *in vitro* (62, 67, 117, 128), but there have been no reports of MEL cell nuclear protein binding *in vitro* to the GATA motif in the mouse ß-min globin promoter nor of proteins binding to the putative GREs in any mouse globin promoters. Therefore, MEL cell nuclear extracts were tested in DNA electrophoretic mobility-shift assays (EMSAs) for their ability to bind to either a radiolabeled oligonucleotide that contained the GATA-RE present in the mouse α_1-globin promoter without a GRE motif or to one that contained both the GATA-RE and the (overlapping) GRE motifs present in the ß-min globin promoter (Fig. 5). No retardation of either probe was observed in the absence of a nuclear extract (not shown here, but see Fig. 6, 7). Two complexes were formed on the α-globin probe (Fig. 5, lane 1). Formation of these complexes was specific since the levels of both complexes were markedly reduced by simultaneous incubation with an excess of either a nonradiolabeled oligonucleotide with the same sequence as the probe (not shown) or a "competitor" 30-mer oligonucleotide containing six tandem AGATA sequences (AGATA multimer), but no complete GRE motifs (lane 2). However, a nonradiolabeled "competitor" 30-mer oligonucleotide containing five repeated GRE hexamer motifs (GRE multimer), but lacking a complete GATA motif, did not inhibit the formation of either complex (lane 3). These findings indicate that *both of the complexes* formed with the *α_1-globin promoter probe* were due to a protein(s) that bound to the GATA site and not to factors binding to the GRE motif. The most rapidly migrating complex may contain a proteolytic fragment of mGATA-1 which still retains the ability to bind GATA-REs as this type of fragment has been noted (59) or, alternatively, the slower migrating complex may contain additional proteins and/or multimers of the same protein (134).

When a MEL cell nuclear extract was incubated with a *ß-min* globin probe two major complexes were seen, but one of these was not equivalent to that formed on the α-

globin probe. The most retarded complex formed with the ß-min globin promoter probe *comigrated* with the GATA-binding complex formed on the α-globin probe, while the other complex formed on the ß-min globin promoter did not comigrate with any complex formed on the α-globin probe, (Fig. 5., *cf.* lanes 1 and 4). The formation of these complexes was specific since coincubation of the ß-min globin probe with an excess of a nonradiolabeled oligonucleotide with the same sequence as the probe eliminated the formation of both complexes (not shown). In addition, coincubation of this probe with an excess of nonradiolabeled AGATA multimer eliminated the slowly migrating complex and reduced, but did not eliminate, the rapidly migrating complex (lane 5 and not shown). On the other hand, coincubation of this probe with an excess of the GRE multimer not only reduced the amount of the rapidly migrating complex, but also somewhat reduced the level of the slowly migrating one (lane 6). Even though the competition by this oligomer at the GATA-binding site was not potent, it was consistent and not entirely expected. The competition at this site appears to be due to the AGA sequence (as recognized on the complimentary strand) that is identical with a portion of the AGATA motif. These results indicate that the slowly migrating complex formed with the ß-min globin probe contained a GATA-binding protein(s) while the rapidly migrating one contained a GRE-binding protein(s) although each synthetic "competitor" oligonucleotide may be able as well to bind at least some portion of each of the two complexes.

The "multimer competitor" oligonucleotides were radiolabeled and their specificties further examined (not shown). Both of these radioactive oligomers formed retarded complexes in EMSAs with MEL cell nuclear extracts. With the AGATA multimer only a single complex formed which comigrated with the one formed at the GATA site on the ß-min probe. This complex was eliminated with an excess of nonradioactive AGATA multimer, but not by an excess of nonradioactive GRE multimer. These results further suggest that the sequence chosen as a GATA-site competitor was specific for this site. In studies of the probe fashioned from the GRE multimer, two signals with the same mobilities as those formed on the ß-min probe were noted: an intense rapidly migrating signal with the mobility of the complex formed with the GRE site and a weak more slowly migrating signal with the mobility of the one formed at the GATA site. An excess of nonradioactive GRE multimer essentially eliminated the rapidly migrating complex and slightly reduced the slowly migrating one. Conversely, an excess of the nonradioactive AGATA multimer eliminated the slowly migrating complex, while only slightly reducing the rapidly migrating one. Therefore, the rapidly migrating complex contained a GRE-binding protein(s) and the less intense signal of the slowly migrating complex was

presumably formed on the AGA sequence noted above. Specific interpretations of EMSAs performed with this sequence as a competitor appeared to be valid, since the signal formed at the putative GATA-site was strong in comparison to that at the GRE site. Therefore, EMSAs were able to distinguish specific binding of GATA-RE-binding factors from GRE-binding ones when the two REs were on the same 30-mer sequence of the ß-min globin promoter. Additional control EMSA experiments were performed utilizing oligonucleotides altered in either the GATA-RE or the GRE motif of the ß-min globin probe. In the former, the sequence of CCCC was substituted for the GATA sequence and in the latter, CCCCCT was substituted for the GRE motif (TCTTGT). These altered oligomers were tested as competitors for MEL cell factors binding to the ß-min globin promoter, AGATA multimer, and GRE multimer probes (not shown). Neither of these altered oligonucleotides were able to compete well at either the GATA or GRE motifs of the ß-min globin probe. The oligomer altered at the GATA site did not inhibit complex formation at the GATA site on the AGATA multimer and neither did nonlabeled oligomer altered at the GRE site. In a similar fashion, excesses of either altered oligomer did not inhibit the formation of a complex on the GRE multimer probe (not shown). Furthermore, retarded complexes were not formed at the GATA or GRE motifs when either of these altered oligonucleotides were labeled and used as probes (not shown). Therefore, the particular alterations made at the GATA or GRE sites were responsible for loss of complex formation at each of these sites.

Effects of induction and inhibition of MEL cell differention on nuclear levels of GATA-RE- and GRE-binding activity

Since GRE- as well as GATA-RE-binding activities were found to be present in MEL cell nuclear extracts, the effects of inducing and inhibiting MEL cell differentiation on these activities were determined (Fig. 6). Incubation of the ß-min probe in the absence of a nuclear extract did not result in a retarded complex (lane 1). Nuclear extracts from untreated cells displayed retarded complexes (lane 10). The addition of excess nonradiolabeled AGATA multimer markedly reduced the amount of the most retarded complex (lane 11) and an excess of the nonradiolabeled GRE multimer eliminated the most rapidly migrating complex (lane 12). Nuclear extracts prepared from cells grown in medium supplemented with charcoal-treated (essentially steroid-free) FBS were not able to form a significant amount of the most rapidly migrating, GRE-binding, complex, but were able to form the slower migrating, GATA-binding, complex (lane 2). Treatment of the cells with DEX markedly increased the level of the GRE-binding complex (lane 4). These findings are consistent with many studies that have demonstrated that DEX acts on GRs in

the cytoplasm aiding in their translocation to the nucleus and their activation [Picard, 1987 #213]. Even extracts prepared from cells treated with DEX for only 1 day displayed nearly a "maximun" level of this GRE-binding complex (lane 6) and further DEX treatment only slightly increased that level (lane 8). Induction of differentiation by HMBA treatment resulted in a modest increase in the level of the presumptive major GATA-1- containing complex, but did not increase the level of the GRE-binding complex (*cf.* lane 3 to 2 and lane 7 to 6). EMSAs containing nuclear extracts prepared from cells treated with HMBA to induce differentiation and concomitant DEX to inhibit the induced differentiation are shown in lanes 5 and 9. Under these conditions, the effect of DEX on the GRE-binding complex was eliminated and the HMBA-induced increase in the major GATA-binding complex was reduced. These results suggest that DEX activated the GR which then interfered with the function of GATA-1 and, moreover, that HMBA treatment reduced the function of the GR. These data are also compatible with the consideration raised above, that the mGR and mGATA-1 might enter into a complex together, possibly prior to establishing DNA contact.

Binding of mGATA-1 to mGR

In order to further test the possibility that mGATA-1 and mGR enter into a protein/protein complex, EMSAs were repeated as in Figures 5 and 6, but following preincubation with either an mGATA-1- or an mGR-specific antibody (Fig. 7). No complexes were formed in incubations containing the DNA probe without the addition of nuclear extract (lane 1) and two complexes representing GATA-RE- (the most intense and most retarded complex) and GRE-binding (the most rapidly migrating complex) factors when a nuclear extract was added (lane 2). Preincubation with a mGATA-1-specific antibody affected both complexes, *i.e.*, the intensity of the major complex, shown above to contain GATA-binding factor(s), was reduced and the rapidly migrating one containing GRE-binding factor(s) was eliminated (lane 3). Preincubation with an mGR-specific antibody also markedly reduced the level of both of these complexes (lane 4). Control preincubations with either IgG (lane 5) or with rabbit preimmune serum (lane 6) did not alter the pattern of the GATA- or GRE-binding complexes. The findings that each of the two unrelated antibodies, when utilized individually, simultaneously reduced the level of complexes forming over GATA-RE and GRE motifs, implies that a complex(es) containing both mGR and mGATA-1 may occur without involving DNA.

Effects of the GR and deletion mutants of mGATA-1 on globin gene and FLV promoter transactivation

Studies with deletion mutant constructs of mGATA-1 have demonstrated that three domains of mGATA-1 are required for the transactivation of an α_1-globin promoter sequence (67). Essential sequences were found to reside in mGATA-1 within the amino terminal region extending from amino acid residue 1 to residue 110 (deletion construct @110), within an internal region extending from amino acid 249 to 290 that eliminated the carboxyl terminal zinc finger domain (deletion construct @249), and within the carboxyl terminal region extending from the 308th to the last, the 413th amino acid (deletion construct @308) (67). These deletion mutant constructs of mGATA-1 were tested for their ability to transactivate ß-min and -maj globin and the FLV promoters (Fig. 8) as they were previously tested with an α_1-globin promoter sequence (67). As noted in Fig. 4, cultures lacking a transfected GATA-1-expressing plasmid did not efficiently drive the transcription of globin-reporter plasmids (Fig. 8A, line 1, 8B, line 1, 8C, lines 1, 2, and 8D, line 1). The activity of each promoter was stimulated by the presence of wild type (WT) mGATA-1 in the absence of "DEX-activated" GR (Fig. 8A, line 2, 8B, line 2, 8C, lines 3, 4, 10, and 8D, line 2). This stimulation was reduced in the presence of DEX-activated mGR (Fig. 8A, line 2, 8B, line 2, 8C, line 11 and 8D, line 2). The three deletion mGATA-1-expressing plasmids did not stimulate expression of any of the reporter plasmids in the absence, and, where tested, the presence of, "DEX-activated" GR (Fig. 8). The slight "residual" stimulatory activity of these mutant mGATA-1 constructs was also inhibited by "DEX-activated" GR (Fig. 8A, lines 3, 4, 5, B, lines 3, 4, 5, C, lines 5, 6, 7, and 8D lines 3, 4, 5), but since the "residual" levels of activity were low, these findings are probably not significant. A caveat for the interpretation of the data obtained with the GATA deletion plasmids is the possibility that the levels of the proteins produced by these plasmids was not as great as that produced by the WT plasmid, for example, due to low yields or degradation of the transcribed mRNAs. However, the levels of the products of constructs @110 and @308 in monkey kidney cells were the same or greater than that of the WT plasmid as determined by EMSAs and the level of the @249 plasmid product (which could not be assayed by EMSA since it does not bind DNA) was also approximately the same as that of the WT plasmid as determined by Western blotting (67).

Effects of GR mutants on the up-regulation of ß-globin and FLV promoters by mGATA-1

In order to map possible domains of the GR that can affect mGATA-1 function, initial experiments tested four GR constructs in transactivation assays (86): one with a point mutation (Glu_{546} altered to Gly) in the hormone-binding domain that eliminates glucocorticoid-responsiveness (NA), one with a point mutation (Arg_{484} altered to His) in the DNA-binding domain that reduces DNA binding (NB), one with an extensive N-terminal deletion (from the amino acid 1 to 420) which removed the transactivation domain (Dd), and one with a less extensive N-terminal deletion from amino acid 1 to 106 (N1) which still contained some of the transactivation domain, including the acidic region located between amino acid residues 196 and 293. CV-1 cells were transfected with plasmids expressing WT mGATA-1, various constructs of mGR, and either a ß-min, ß-maj, or FLV promoter-driven reporter plasmid in the presence and absence of DEX (Fig. 9). Cultures lacking transfected mGATA-1 in the absence or presence of DEX did not stimulate the activity of either the ß-globin or FLV promoter (line 1, Fig. 9A, B, and C). The expression of reporter plasmids in cultures transfected with mGATA-1, but not with mGR, were stimulated over 15-fold in the absence and presence of DEX (line 2, Fig. 9A, B, and C). Cultures transfected with mGATA-1 and WT mGR stimulated the activity of both reporter plasmids over 15-fold in the absence of DEX, but in the presence of DEX the stimulation was reduced over 70% in each case (line 3, Fig. 9A, B, and C). The NB construct responded to DEX as well as the WT mGR (line 5, Fig. 9A, B, and C) indicating that the DNA binding function is not required for this activity of the GR. The three other GR mutant constructs tested, which were deficient in either their hormone-binding or N-terminal domains, all lacked responsiveness to DEX and, therefore, are altered in critical regions (lines 4, 6, 7, Fig. 9A, B, and C). As noted above for the GATA deletion constructs, it is possible that the levels of products expressed from the GR deletion constructs might not have been as great as those from the WT-expressing plasmid. This was not the case for the NB plasmid since its product responded to DEX treatment like the product of the WT plasmid. It is also known that the mRNAs expressed from the NA and N1 plasmids are stable in monkey kidney cells (86) and the result obtained here with the Dd plasmid was essentially the same as that found for the N1 construct. However, there is a possibility that either inefficient translation of a product mRNA or proteolytic activity affecting one of the construct products to a greater degree than that of the WT plasmid might have occurred.

DISCUSSION

GATA-1 stimulates the expression of both mouse ß-minor and ß-major proximal globin gene promoter-driven reporter plasmids

It has been shown here that the GATA motifs located about 200 bp 5' of the cap-site in the mouse ß-maj and ß-min globin gene and FLV promoters (Fig. 1) are stimulated by mGATA-1 (Fig. 3). These findings strengthen the idea that GATA-1 regulates erythroid-specific gene expression and along with the demonstrations that functioning GATA-REs are similarly located in other erythroid genes, *i.e.*, in the mouse α_1-globin (67) and erythropoietin receptor (81) genes, they suggest that this is an apparently "preferred erythroid" location for a GATA-RE. Further support for this idea may be forthcoming from studies of similarly located GATA motifs present in some *non-globin*, erythroid-specific genes, *e.g.*, porphobilinogen deaminase (PBGD) (135, 136), aminolevulinate dehydratase (ALAS) (137), and aminolevulinate synthase (ALAS) (138). Moreover, functional studies of mGATA-1 domains required for transactivation of mouse ß-globin promoters (Fig. 8A, B, and C), indicate that they are the same regions of the protein shown previously to be required for transactivating the mouse α_1-globin and a chicken globin promoter GATA-RE (67, 134, 139). Insofar as it was tested, intact mGATA-1 is required for transactivation since deletions in the N-terminal, the carboxyl terminal zinc finger, or in the C-terminal domain eliminate the mGATA-1 stimulation of the GATA-RE in both mouse ß-globin promoters (Fig. 8A, B, and C).

GATA-1 stimulates the expression of erythrotropic viral promoters

The present study also demonstrates that a promoter-containing sequence derived fromFLV can be up-regulated by GATA-1 (Fig. 3C). The promoter contained a GATA motif (Fig. 1) which probably accounts for the responsiveness to GATA-1. As noted aboved, two regions of the FLV genome, the LTR and the region coding foe the gp55, are known to be required for both the erythrophilia and pathologic effects of FLV. The mechanism of the gp55-coding region has been explained, but not that of the LTR. The response of the LTR to GATA-1 observed here appears to resolve this issue. Moreover, functional studies of mGATA-1 domains required for transactivation of mouse FLV LTR (Fig. 8D), indicate that they are the same regions of the protein shown previously to be required for transactivating the mouse α_1-globin and a chicken globin promoter GATA-RE (67, 134, 139). That is, deletions in the N-terminal, the carboxyl terminal zinc finger, or in the C-terminal domain eliminate the mGATA-1 stimulation of the GATA-RE in both mouse FLV promoters (Fig. 8D). The specific nucleotides required for the GATA-1 response can

now be determined by studies utilizing LTR-reporter plasmids containing alterations in the GATA-RE prepared by site-directed mutagenesis and by DNA footprinting assays.

Mechanism of the mGR modulation of mGATA-1 function

The inhibition of mGATA-1 activity by "glucocorticoid-activated" GR was characterized by demonstrating that the level of inhibition was directly related to the presence of a GR-expression plasmid and to the concentration of DEX for all four WT reporter plasmids examined (Fig. 4) and by initial mapping studies with reporter plasmids expressing altered forms of the GR (Fig. 9). Plasmids expressing three different forms of the GR (NA, Dd, N1) mutant in different portions of the coding region were shown not to be able to mediate an inhibition of mGATA-1 function and one altered form of GR (NB) was able to do so (Fig. 9). The NA mutant presumably did not respond to DEX and mediate the glucocorticoid-dependent inhibition of mGATA-1 transactivation because this mutant lacks a hormone-binding domain, has reduced affinity for glucocorticoid, and cannot translocate to the nucleus to take part in gene regulation (140). The Dd mutant lacking the entire 421 amino acid N-terminus also did not alter mGATA-1 function. This region might well be responsible for an interaction with mGATA-1 since it plays important roles in transactivation and protein/protein interactions (86, 93). Control of transactivation by this region is thought to be due to the presence of an acidic transactivating domain (which appears to place the GR in the acidic class of transactivators) [Eriksson, 1990 #197; McEwan, 1993 #233] and to phosphorylation sites which are known to modify the ability of the GR to mediate transactivation (141-143). The N1 mutant construct, which lacks the N-terminal 106 amino acids, also was unable to mediate the DEX-dependent inhibitory effect. This region of the mGR overlaps the τ_1 transactivation domain of the human GR positioned between amino acids 77 and 262, see (144), so that a critical sequence may occur within the relatively short stretch of amino acids from residue 77 to 106. Further mapping of this function utilizing deleted and mutated constructs as well as by transfection with constucts containing only sequences of the N-terminal domain are planned. Since the construct utililized contains 91% of the transcriptional activity of the WT GR and the same level of hormone-binding affinity as the WT GR (86), a function must exist in this N-terminal region that is required for DEX-mediated down-regulation, but not required for GR-mediated transactivation. Therefore, these two glucocorticoid-dependent activities, of the GR, *i.e.*, inhibition of GATA-1 function and transactivation of GR-responsive genes, are not mediated in the same fashion. This is further emphasized by the finding that the NB construct, which is mutated in its DNA-binding domain and will not optimally transactivate

a response element (86), was still able to mediate the DEX-dependent inhibition of mGATA-1 transactivation studied here. Taken together, these results imply that the DEX-dependent inhibition of mGATA-1 function may occur in the absence of a GRE due to a direct or indirect protein association of mGATA-1 and GR and that this association may involve the N-terminal region of the GR.

Many transactivating factors act in concert with other proteins as homo- or heterodimers or multimers (145). In particular, the GR has been shown to act with and/or bind to other transcription factors such as AP-1 (145-152) and octamer transcription factor 1 (153). However, the results presented here provide the first example of an interaction of GR with GATA-1 and apparently represent the first evidence of a functional interaction of any transcription factor with GATA-1, although the latter has been considered (59). Of four possible direct types of interactions between mGATA-1 and activated mGR that might be responsible for the down-regulation of mGATA-1 activity by mGR that can be envisioned (Fig. 10), the fourth possibility, a complex of mGATA-1 and activated mGR not bound to a response element, appears to be the most likely although the first and third possibile interactions may also occur. These considerations would still apply if any of these hypothesized complexes included a protein(s) in addition to mGATA-1 and the mGR. To further examine the possibility of mGATA-1 and mGR interactions, DNAase protection assays and experiments designed to crosslink any proteins that may be associated with either the mGR or mGATA-1 *in vivo* are in progress.

The results presented (4, 154, 155) here not only suggest that a protein/protein interaction involving GATA-1 and the GR appears to be the most likely mechanism explaining the DEX-mediated down-regulation of GATA-1 function, the glucocorticoid-mediated inhibition of induced MEL cell differentiation (Fig. 7), and induced reverse transcriptase activity (1), but that the GRE hexamer motifs present in globin and other erythroid promoters may be responsible for intensifying these effects of DEX. Two findings support the latter contention: 1) Binding *in vitro* to GRE sequences neighboring GATA-REs has been demonstrated (Fig. 5-7) and 2) the presence of this motif in the promoter region augmented DEX-mediated inhibition of mGATA-1 function (Fig. 4).

Possible mechanisms for the glucocorticoid-mediated inhibition of MEL cell differentiation and induced reverse transcriptase activity

Two of the more intriguing (but not mutually exclusive) mechanisms for the glucocorticoid-mediated inhibitory effects in MEL cells are the interference by activated GR of structral and/or regulatory erythroid genes. The erythroid structural genes, globins and FLV-coded products, and possibly other genes that were not studied that cintain GATA-1 promoter sequence such as ALAS, ALAD, PBGD, EpoR, are known or postulated here to be GATA-1-regulated and therefore may all be down-regulated by activated GR. However, down-regulation of the erythroid regulatory gene, GATA-1, is also possible. Undifferentiated MEL cells contain a high level of GATA-1 mRNA and GATA-1 which increase further during DMSO- and HMBA-induced differentiation as reported by others (59, 62, 117), confirmed here (Fig. 6), and also observed during induction of erythrodifferentiation in a human cell line, K562 (156). The importance of the increase in GATA-1 levels during erythroid development has been emphasized in a recent review (157). Two methods were used here to examine the effect of DEX treatment of MEL cells on nuclear levels of GRs: EMSAs and ^{3}H-DEX binding. When MEL cells were treated with DEX, there was an increase in the binding of nuclear components to GRE motifs (Fig. 6) and an increase in the ability of nuclear extracts to specifically bind ^{3}H-DEX, *i.e.*, the binding was "competed-out" by an excess of non-radiolabeled DEX (not shown). In cells treated simultaneously with HMBA and DEX to reduce the level of induced hemoglobin synthesis (2, 4-14, and Fig. 2), the functions of both GATA-1 and the GR also were decreased (Fig. 6). A mechanism, based upon the autostimulatory ability of GATA-1, provides an explanation for the observed global inhibition of MEL cell erythrodifferentiation by glucocorticoids. GATA-1 is able to up-regulate the transcription of its own gene due to a GATA-RE located in its promoter (82, 134). Therefore, the activation of GRs may lead not only to decreased transcription of globin and other erythroid-specific, GATA-RE-containing genes for structural products, but also to a decrease in transcription of an erythroid regulatory gene product, GATA-1 itself. A decrease in the transcription of this gene would lead to decreases in transcription of all of the erythroid-specific genes it regulates. The GR could affect the autoregulation of mGATA-1 due to protein-protein interactions, but, as postulated for the globin promoters, an additional effect may be mediated through a GRE. In this regard, the double GATA motif, which acts as a GATA-RE in the mGATA-1 promoter (82), contains within itself a perfect, primary hexamer GRE motif with the exception of a single mismatched base-pair. It will be of interest to test the ability of this GRE motif to react with the GR.

In summary, it is shown here that mGATA-1 stimulates expression of mouse ß-globin genes and the FLV LTR in a similar fashion to that of the mouse α_1-globin gene,

and that "activated" GR inhibits this stimulation. These studies place both ß-globin promoters and FLV LTR in a growing group of GATA-1-regulated sequences. In addition, they appear to partially explain the mechanism of the erythrotropism of the FLV LTR and the glucocorticoid down-regulation of induced MEL cell differentiation and retroviral reverse transcriptase activity levels. Experiments with reporter plasmids containing different promoter sequences as well as studies with GATA-1- and GR-specific antibodies indicate that the mGR may affect mGATA-1 function due to a protein-protein interaction. Studies with mutant constructs of mGR which lack the ability to inhibit globin gene and FLV expression suggest that a region in the N-terminal 106 amino acids is involved in this interaction. A GRE motif nearby a GATA-RE may intensify GR-mediated inhibition. Since GATA-1 up-regulates its own transcription, an interaction of mGATA-1 and the mGR could explain the DEX-mediated inhibition of all MEL cell GATA-1-regulated, erythroid gene expression although DEX-mediated inhibiton of GATA-1-regulated structrual genes also comfortably explains the inhibition.

MATERIALS AND METHODS

PLASMIDS

An mGATA-1 cDNA-expressing plasmid (a 1.8 Kb cDNA *Xho*I insert in the mammalian expression vector, pXM) and a series of mutant mGATA-1 plasmids @110 , @249, @308, deleted from amino acid 1 to 110, 249 to 290, and 308 to the end, which eliminate the N-terminal region, a critical zinc finger-DNA-binding region, and the C-terminal region, respectively (59), and a reporter plasmid containing a synthetic 20-mer α_1-globin promoter including a GATA-RE octamer with six flanking nucleotides (GGGCAACTGATAAGGATTCC) on each side inserted into a human growth hormone (HGH)-plasmid were obtained from S.H. Orkin. A 550 bp HincII ß–maj globin fragment containing globin regulatory sequences (5'-promoter and cap-site) inserted into the HindIII site of a PSV-CAT plasmid, a 702 bp BamHI-HincII ß-min globin fragment fused into the HindIII site of PSV-CAT plasmid, and a 700 bp NcoI α_1–globin fragment fused into the HindIII site of a PSV-CAT (126) were obtained from M. Sheffery. A CAT-expressing plasmid containing a 5'-sequence consisting of a Sau3A-KpnI approximately 380 bp fragment of the FLV LTR (158) was obtained from E. Golemis. The wild-type mGR cDNA expression plasmid and the series of GR mutant plasmids, NA (a hormone-binding domain point mutant, Glu_{546} to Gly), NB (a DNA-binding domain point mutant, Arg_{484} to

His), Dd (an N-terminal mutant lacking amino acids 1 to 421), and N1 (an N-terminal mutant lacking amino acids 1 to 106) were obtained from M. Danielsen (140).

CELL CULTURE

MEL cell line DS-19 (33) at the 100-160 passage, was grown in Glasgow minimal essential medium (BHK) (GIBCO) with 15% fetal bovine serum (FBS) (HYCLONE). The cell number was determined with a Zf particle counter with an attached channelyzer and XY plotter (COULTER Electronics, Inc., Hialeah, FL). MEL cells were induced to differentiate along the erythroid pathway by adding 5 mM HMBA (Sigma Chemical Co., St. Louis, MO) to the culture medium for 4 or 5 days (unless noted) and then monitored for the presence of both hemoglobin-containing (benzidine-positive) and viable, trypan blue-excluding, cells (3, 24). CV-1 cells were obtained from ATCC and grown in DMEM medium (GIBCO) with 10% FBS (HYCLONE). Steroid-reduced serum (159, 160) was prepared by mixing 100 ml serum with 0.5 gm activated charcoal (EM Science) at 4°C overnight and then passing it through a Nalgene 45 μ filter. Because commercial serum present in the cell culture medium contains significant amounts of steroids and possibly other compounds (161) that can influence the activity of transacting factors such as glucocorticoid receptors, experiments were generally performed with charcoal-treated FBS in place of untreated FBS to reduce the levels of these possible "contaminants" (159, 160).

CELL TRANSFECTION

CV-1 cells were seeded at a density of 10^6 cells per 10 cm dish, grown for a day, then 3 hr before transfection the medium (DMEM) containing 10% steroid-free FBS was changed. For each dish, 10 μg of a CAT-containing plasmid, 5 μg of plasmid pCH110 coding for ß-galactosidase (Pharmacia) and 10 μg of each expression plasmid were cotransfected with calcium phosphate added to the medium according to a standard procedure (54). Sixteen hours later the medium was exchanged for fresh steroid-free medium and DEX (1 μM) added where indicated. Forty-eight hours after transfection, cells were harvested, washed with PBS, pelleted, and resuspended in 100 μl of 0.25 M Tris.HCl, pH 7.8. Total cell extracts were prepared utilizing 3 freeze-thaw cycles followed by centrifugation. The resulting supernatant fluids were tested for the presence of GR by determining the level of specific [^{3}H] DEX-binding, *i.e.*, binding that can be "competed-out" by non-radiolabeled DEX (162). Only supernatant fluid samples derived from cultures transfected with the mGR-expressing plasmid demonstrated specific [^{3}H] DEX-binding (data not shown).

Supernatant fluid samples were also tested for the presence of mGATA-1 by Western blotting with an anti-mGATA-1 antibody: cell extract protein samples (100 μg) were electrophoresed on 10% polyacrylamide/sodium dodecyl sulfate gels and the proteins were transferred to filters that had been "blocked" by incubation in BSA (1%) overnight at 4°C. The following procedures were performed at room temperature. The filters were washed twice with T/S/T (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) for 5 min and then incubated for 90 min with anti-GATA-1 antibody, obtained from S.H. Orkin, (diluted 1:1000 in T/S/T plus 1% BSA). The filters were further washed and developed using the Proto-blot system (Promega, Madison, WI) containing an anti-IgG alkaline phosphatase conjugate. Only samples derived from CV-1 cultures transfected with the mGATA-1-expressing plasmid were found to contain mGATA-1 (data not shown). Then the posttransfection supernatant fluid samples were assayed for ß-galactosidase activity as a transfection level control (163) and for CAT or HGH activity.

To assay CAT activity (164), a volume of cell extract containing 30 units of ß-galactosidase activity was mixed with 80 μl of the CAT reaction mixture: 50 μl of 1 M Tris·HCl, pH 7.8, 10 μl of ^{14}C-labeled choramphenicol, 0.1 mCi/ml (New England Nuclear Research Products, Boston, MA), 20 μl acetyl coenzyme A (3.5 mg/ml in H_2O) and incubated at 37°C for 1 hr. Ethylacetate (1 ml) was added to the mixture which was then vortexed and centrifuged. The supernantant fluid samples were vacuum-dried and resuspended in 15 μl of ethylacetate, spotted on a silica gel thin layer plate and chromatographed in chloroform-methanol (19:1) for 1 hr to separate native chloramphenicol from its acetylated derivatives. After autoradiography overnight, the spots were scraped off and their radioactivity determined in order to quantitate the amount of chloramphenicol converted into acetylated forms. The CAT activity was expressed as the fold-increase over a base-line value of an acetylated form of chloramphenicol.

Growth hormone levels were determind according to the Allégro radioimmunoassay kit protocol (Nichols Institute, San Juan Capistrano, CA). The medium of CV-1 cells 48 hr after HGH plasmid transfection was directly assayed for growth hormone activity. Medium (100 μl) was incubated with 100 μl of ^{125}I-antibody solution and beads at room temperature for 4 h. The beads were washed twice and the radioactivity levels were determined with a gamma counter.

NUCLEAR EXTRACT PREPARATION

Nuclear extracts were prepared according to the procedure of K. Ullmann and G. Crabtree (Stanford University Medical School, CA, personal communication) with slight modifications. All procedures were performed at 4°C. Cells were collected by centrifugation, washed in phosphate-buffered saline (PBS, GIBCO) containing Ca^{2+} and Mg^{2+} and once in PBS without these cations, and suspended in 1 ml lysis buffer A [10.0 mM HEPES, pH 7.8, 15.0 mM KCl, 2.0 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT), 0.1 mM EDTA, 1.0 mM phenylmethanesulfonyl flouride (PMSF), 0.2% nonidet P 40 (NP-40), and 1 μg/ml each of antipain, leupeptin, N-tosyl-L-phenylalanine (TPCK), and diisopropyl fluorophate (DFP). The suspension was centrifuged and the nuclear pellet was resuspended in 150 μl of lysis buffer B [50.0 mM HEPES, pH 7.8, 50.0 mM KCl, 0.1 mM EDTA, 1.0 mM DTT, 10% glycerol, 1.0 mM PMSF, and 1 μg/ml each of antipain, leupeptin, TPCK, and DFP]. Nuclear proteins were precipitated by adding an ammonium sulfate solution to a final concentration of 0.3 M and the supernatant fluid was obtained after centrifuging in a TLA 100.3 rotor in a Beckman TL100 centrifuge at 70K rpm for 15 min. Ammonium sulfate was added to the supernatant fluid to a final concentration of 1.8 M and the suspension was centrifuged at 50 K rpm for 10 min. The pellet was suspended in buffer B, assayed for protein concentration with Bio-Rad protein assay reagent utilizing bovine serum albumin (BSA) as a standard, and stored in aliquots at -80°C.

DNA ELECTROPHORETIC MOBILITY-SHIFT ASSAY (EMSA)

Ten μg of nuclear extract was incubated with 1 μg each of salmon sperm DNA and poly dI-dC in binding buffer [10.0 mM Tris·HCl, pH 7.5, 50.0 mM NaCl, 1.0 mM EDTA, 1.0 mM DTT, 50.0 μg/ml BSA, 2.0 mM $MgCl_2$, and 5% (v/v) glycerol] in a total volume of 20 μl for 20 min at room temperature, followed by a 30 min incubation at room temperature with a radiolabeled oligonucleotide probe containing 10,000 cpm [the (+) strand and its complementary (-) strand were synthesized on an automated solid-phase synthesizer, purified by polyacrylamide gel electrophoresis, and the (+) strand was 5'-end-labeled with γ-^{32}P-ATP, and then annealed with the unlabeled (-) strand]. Then the reaction mixture was loaded onto a 5% non-denaturing polyacrylamide gel (acrylamide to bisacrylamide ratio of 29:1) containing 1 x TBE (0.09 M Tris·Cl, 0.09 M borate, 2.0 mM EDTA). Gels were electrophoresed for 1.5 hr at 10 V/cm, then dried and subjected to autoradiography.

CHARACTERIZATION OF PROTEINS IN DNA-BINDING COMPLEXES BY ANTIBODY PRECEPITATION

Protein A-Sepharose CL-4B was prepared for use by swelling 15 min in 0.1 M phosphate buffer, pH 7 and washing twice with the same buffer and stored at 4°C according to the manufacturer's instructions (Pharmacia, Piscataway, NJ). Rabbit anti-mGATA-1-specific antibody (58) and anti-mGR-specific monoclonal antibody (MAB) were obtained from S.H. Orkin and Affinity BioReagents, Neshanic Station, NJ prepared as described (165), respectively. The prepared protein A-Sepharose suspension (200 µl) was incubated with 100 µl of each antibody sample with gentle agitation on a rocking shaker for 1 h at room temperature (166). Each protein A-Sepharose-treated antibody sample was then incubated with 15 µg nuclear extract protein and constant agitation overnight at 4°C and then centrifuged. Then the supernatant fluid samples were analyzed by EMSA.

Figure legends

Fig. 1. Sequences of portions of the mouse globin gene promoters studied and the FLV LTR. GATA-REs and GREs are underlined. Sequences utilized as probes are indicated by stars.

Fig. 2. Charcoal treatment of FBS alters the effects of HMBA and DEX on MEL cell differentiation. HMBA and DEX were added, where indicated, at 5 mM and 35 μM, respectively. MEL cells were seeded at 10^5/ml in media supplemented with untreated FBS, cultured for one day to establish logarithmic growth, centrifuged, and then cultured for a total of 6 days under the following conditions. Growth of cells in Group 1 was continued throughout the experiment in the same type of medium. After the first and second days of culture the cells were centrifuged and resuspended in the same volume of fresh medium. On the second day the medium was supplemented with: lane 1, no agents, lane 2, HMBA, lane 3, DEX, and lane 4, HMBA and DEX and the cultures continued for 4 days. The cells in Groups 2 and 3 were grown for the experiment in medium supplemented with charcoal-treated FBS. After 1 day of culture the cells were centrifuged and resuspended in the same type of medium without (Group 2) or with the addition of DEX (Group 3). After a second day of culture, the cells were centrifuged again and resuspended in media supplemented with: lane 1, no agents, lane 2, HMBA, lane 3, DEX, and lane 4, HMBA and DEX and the cultures continued for 4 days. Then the percentages of hemoglobin-containing (benzidine-positive, B^+) cells were determined as described in Materials and Methods. Neither HMBA nor DEX treatment reduced cell yields or viability (not shown).

Fig. 3. The effects of mGATA-1 on globin gene promoters and FLV LTR transactivation. CV-1 cells were cotransfected with ß-maj (diamonds), ß-min (open squares), or α_1- (filled squares) globin promoter sequences linked to CAT reporter plasmids (A) or a synthetic 20-mer α_1- globin promoter sequence lacking a GRE motif linked to a human growth hormone (HGH) reporter plasmid (B) or FLV LTR sequence linked to CAT reporter plasmid (C) described in Materials and Methods, and various amounts of an mGATA-1-expressing plasmid as indicated. After 48 hr, the CV-1 cells were harvested and CAT levels determined or 100 μl of medium were collected and HGH levels determined. Data are expressed as the fold-increase over the values obtained in the absence of transfection with the mGATA-1-expressing plasmid. All the experiments presented here have been performed in duplicate and repeated at least two times with similar results.

Fig. 4. Interference of mGATA-1 transactivation by "DEX-activated" mGR. As in Fig. 3, CV-1 cells were cotransfected with ß-maj (diamonds), ß-min (open squares), or α_1- (filled squares) globin-linked to CAT plasmids (A) or a synthetic 20-mer α_1- globin promoter sequence lacking a GRE motif linked to HGH reporter plasmid (B) or FLV LTR sequence linked to CAT reporter plasmid (C) and mGATA-1- and mGR-expressing plasmids as indicated. Various concentrations of DEX were added as indicated 16 hr after transfection. After a total of 48 hr, the cells were harvested and CAT levels determined or 100 µl medium were collected and HGH levels determined. Data are expressed as the fold-increase over the values obtained in the absence of transfection with the mGATA-1-expressing plasmid.

Fig. 5. Binding of MEL cell nuclear factors to GATA-RE and GRE motifs in mouse globin promoters. MEL cell nuclear extracts were prepared, incubated with a ^{32}P-labeled probe, and EMSAs were performed as described in Methods. The incubations contained: (lanes 1-3), nuclear extracts and the 30-mer α_1-globin promoter sequence probe (shown in Fig. 1), (lane 2), an 100-fold excess of non-radiolabeled "competitor" GATA-RE-containing 30-mer oligonucleotide, (lane 3), an 100-fold excess of non-radiolabeled "competitor" GRE-containing 30-mer oligonucleotide composed of five repeats of the sequence TCTTGT, (lanes 4-6), the 30-mer ß-min globin labeled ^{32}P-probe (shown in Fig. 1), (lane 5), an 100-fold excess of non-radiolabeled "competitor" GATA-RE-containing oligonucleotide, and (lane 6), an 100-fold excess of non-radiolabeled "competitor" GRE-containing oligonucleotide.

Fig. 6. Effects of induction and inhibition of MEL cell differention on nuclear levels of GATA-RE- and GRE-binding activities. Nuclear extracts were prepared (see Materials and Methods) from MEL cells grown as in Fig. 2 The extracts of Group A, B, and C were prepared from cultures grown as in Fig. 2, Group 2, 3, and 1, respectively. The extracts were incubated with the ^{32}P-labeled ß-min globin promoter probe (shown in Fig. 1) and EMSAs were performed as described in Materials and Methods. All EMSA incubations contained the ß-min globin promoter probe, and (lane 1) no nuclear extract, (lanes 2, 6, 10) nuclear extracts from cells not exposed to DEX or HMBA, (lanes 3, 7) extracts from HMBA-treated cells, (lanes 4, 8) extracts from DEX-treated cells, (lanes 5, 9) extracts from HMBA- and DEX-treated cells, (lanes 11, 12) extracts from cells not exposed to DEX or HMBA were preincubated with an 100-fold excess of "competitor" non-radiolabeled GATA-RE- (lane 11) or GRE- (lane12) containing oligonucleotides,

respectively. The upper and lower arrows indicate GATA- and GRE-binding complexes, respectively.

Fig. 7. Antibody characterization of protein binding to a ß-min promoter region sequence. Protein A-Sepharose or a complex of protein A-Sepharose with mouse IgG_{2A}, rabbit preimmune serum, or an antibody to mGATA-1 or to mGR was incubated with a MEL cell nuclear extract. Then EMSAs were performed with the ^{32}P-labeled ß-min globin probe as described in Fig. 6 and Materials and Methods. All of the samples, except in lane 1, contained MEL cell nuclear extract. Lane 2 contained nuclear extract incubated with Protein A-Sepharose, but not an antibody preparation, lane 3, mGATA-1-specific antibody, lane 4, mGR-specific MAB, lane 5, mouse IgG_{2A}, and lane 6, rabbit preimmune serum.

Fig. 8. Effects of the GR and deletion mutants of mGATA-1 on globin gene promoter and FLV LTR transactivation. As in Fig. 3, CV-1 cells were transfected with ß-min (A), and ß-maj (B) globin-linked to CAT plasmids, or a synthetic 20-mer α_1- globin promoter sequence lacking a GRE motif linked to HGH reporter plasmid (C) or FLV LTR sequence linked to CAT reporter plasmid (D). Ten µg of the mGR-expressing plasmid was cotransfected (except in indicated lanes of Panel C) along with 10 µg wild type (WT) mGATA-1 or a truncated mGATA-1 construct; @110, @249, and @308, as indicated, and 16 hr after transfection, DEX, 1 µM, was added to the indicated samples. After a total of 48 hr, the cells were harvested and CAT levels determined (A, B, and D) or 100 µl medium were collected and HGH levels determined (C). Rectangular cartoons of the mGATA-1 portion of these constructs are pictured to the left with the numbers representing the position of amino acids. The filled squares within the rectangular bars indicate the zinc finger domains.

Fig. 9. Effects of mGR mutant constructs on the regulation of ß-globin promoters and FLV LTR by mGATA-1. CV-1 cells were cotransfected with ß-min (A), the ß-maj (B) globin-linked to CAT plasmids, or FLV LTR sequence linked to CAT reporter plasmid (C) as described in Fig. 2 and mGATA-1-and WT mGR- and four different mutant mGR-expressing plasmids as indicated; one with a point mutation (Glu_{546} altered to Gly) in the hormone-binding domain (NA), one with a point mutation (Arg_{484} altered to His) in the DNA-binding domain (NB), one with an extensive N-terminal deletion (from amino acid 1 to 421) which removed the transactivation domain (Dd), and one with a less extensive N-terminal deletion of the transactivation domain from amino acid 1 to 106 (N1). DEX, 1

μM, was added to the indicated samples 16 hr after transfection. After a total of 48 hr, the cells were harvested and CAT levels determined. Rectangular cartoons of the mGR portion of these constructs are displayed to the left. The box enclosed within the rectangular bars indicates the DNA-binding domain.

Fig. 10. Four possible types of interactions between mGATA-1 and activated mGR that might be responsible for the down-regulation of mGATA-1 activity by mGR. (1) A complex of activated mGR bound to mGATA-1 and to both response elements. (2) A complex of mGATA-1 and activated mGR bound to a GATA response element. (3) A complex of mGATA-1 and activated mGR bound to a GRE. (4) A complex of mGATA-1 and activated mGR not bound to a response element. Any of these hypothesized complexes could include a protein(s) in addition to mGATA-1 and the mGR. The oval represents GATA-1, the notched square, the GR, the small black square, a glucocorticoid, the rectangular bar, a mGATA-1-responsive gene, and the dark and hatched rectangles enclosed within the bar represent a GATA-RE and GRE, respectively.

ß-minor globin

```
5'---CTGCGAGGATAAGAACAGACACTACTCAGA---154bp-TATA-25bp-CAP3'
3'---GACGCTCCTATTCTTGTCTGTGATGAGTCT----------------------5'
     ****************************
```

ß-major globin

```
5'--GACAGTGTTCTCTGCACAGATAAGGACAAACATTATT-162bp-TATA-26bp--CAP3'
3'--CTGTCACAAGAGACGTGTCTATTCCTGTTTGTAATAA--------------------5'
      ****************************
```

α1-globin

```
5'-GTCCGGGCAACTGATAAGGATTCCCTG-134bp-AGGACA-14bp-TATA-29bp-CAP3'
3'-CAGGCCCGTTGACTATTCCTAAGGGAC-------TCCTGT------------------5'
```

α1-globin probe

```
TGTGTCCGGGCAACTGATAAGGATTCCCTG
ACACAGGCCCGTTGACTATTCCTAAGGGAC
******************************
```

FLV-LTR

```
5'---GGGCCAAGAACAGATACGCTGGGCCAA---120bp---TATA---24bp---CAP3'
3'---CCCGGTTCTTGTCTATGCGACCCGGTT---------------------------5'
       ********************
```

Fig. I

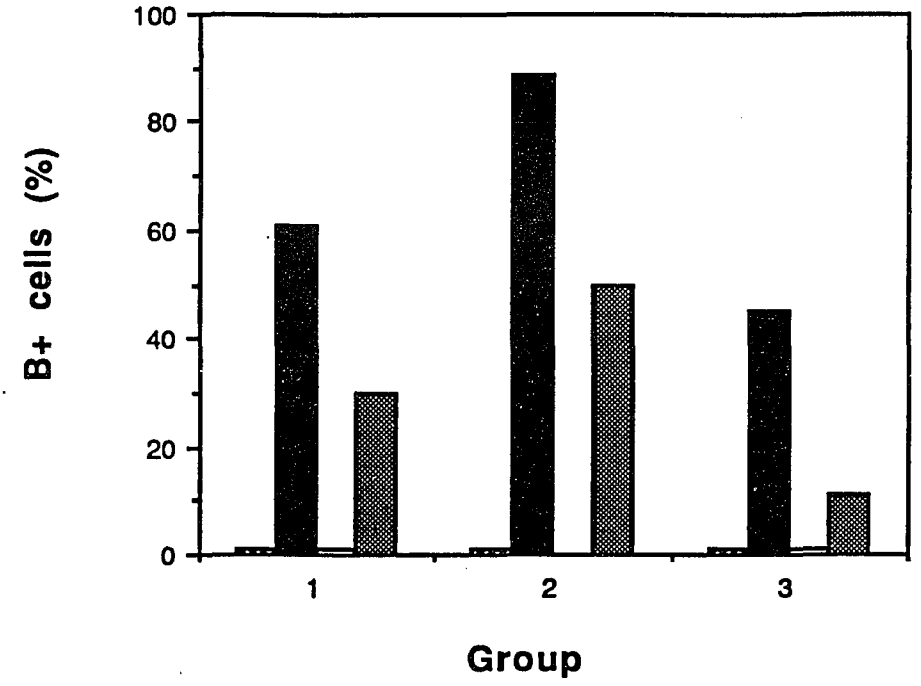
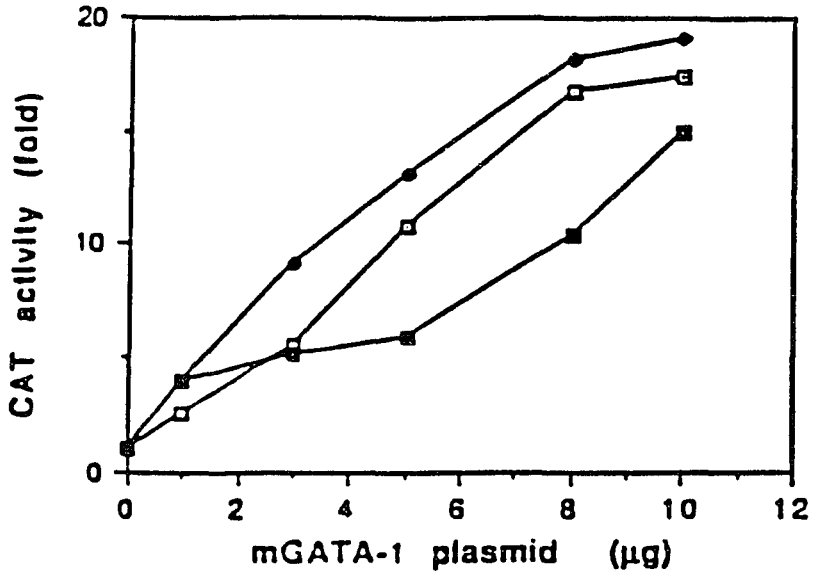


Fig.2

A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10
0
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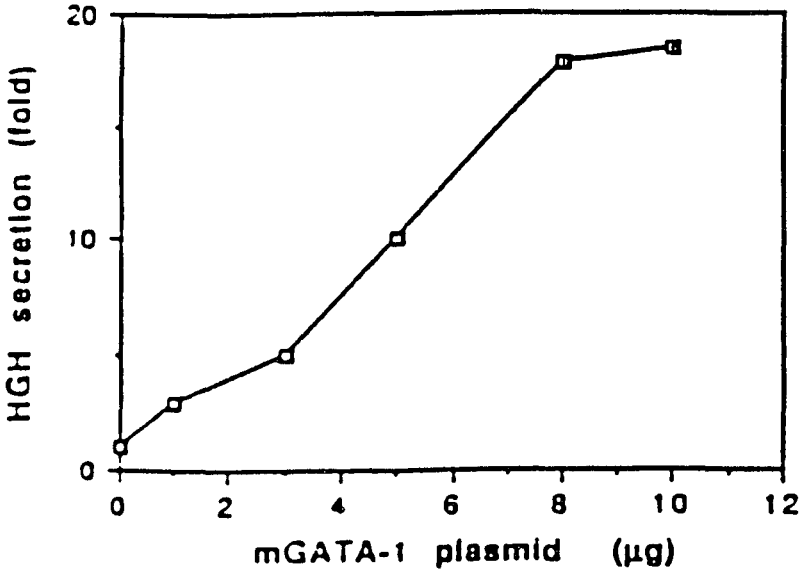
0
2
4
6
8
10
12
mGATA-1 plasmid (μg)

B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10
0
HGH secretion (fold)
0
2
4
6
8
10
12
mGATA-1 plasmid (μg)

Fig.3

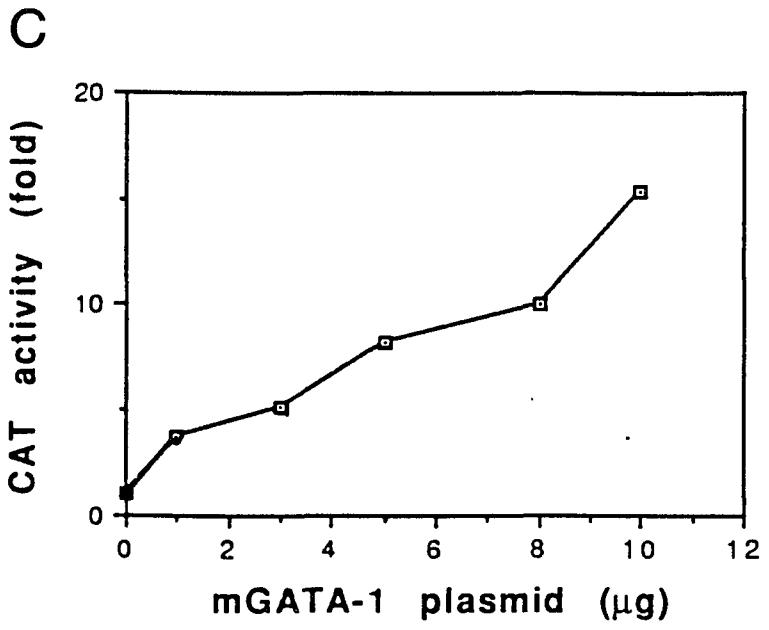
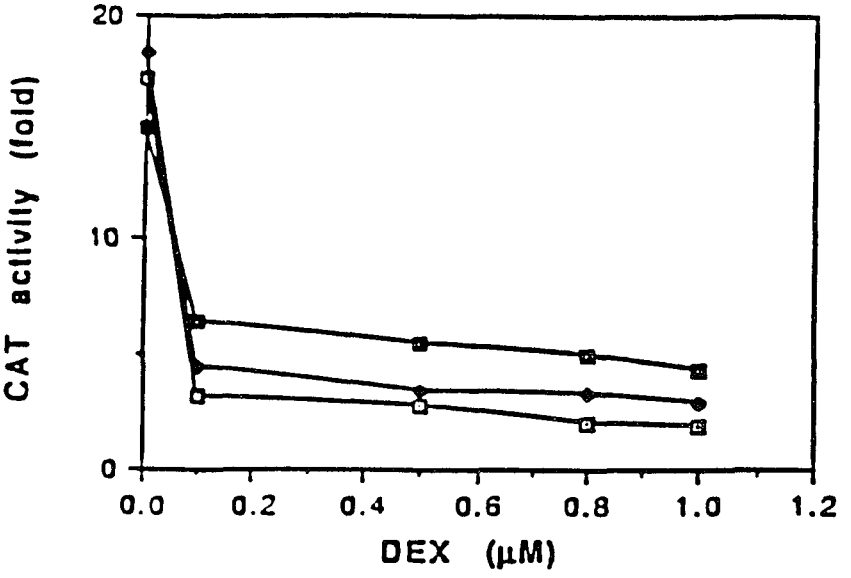


Fig.3

A


CAT activity (fold)
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10
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
DEX (μM)

B

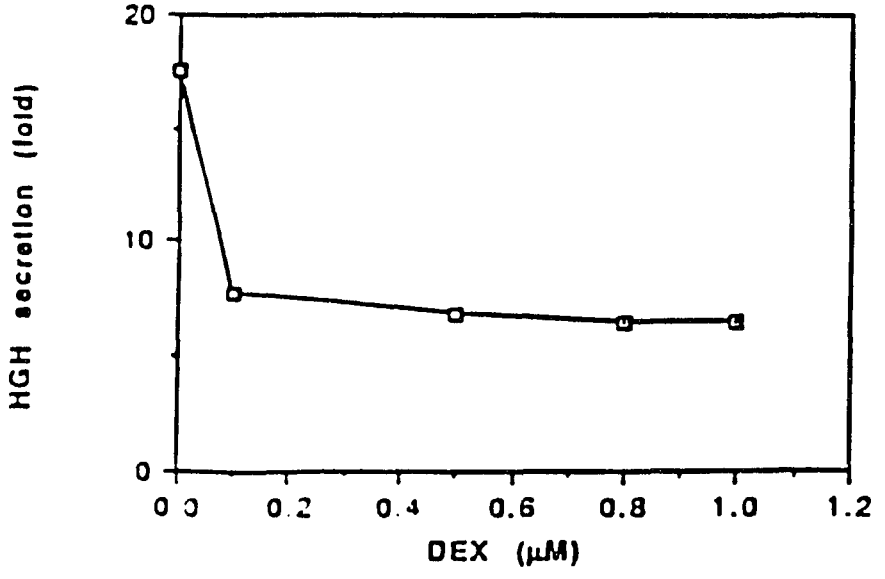

HGH secretion (fold)
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
DEX (μM)

Fig.4

C

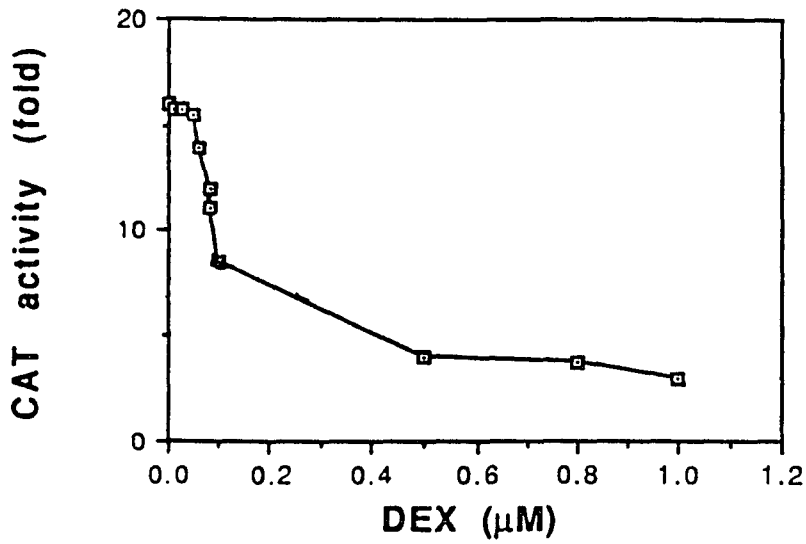


Fig. 4

Fig.5

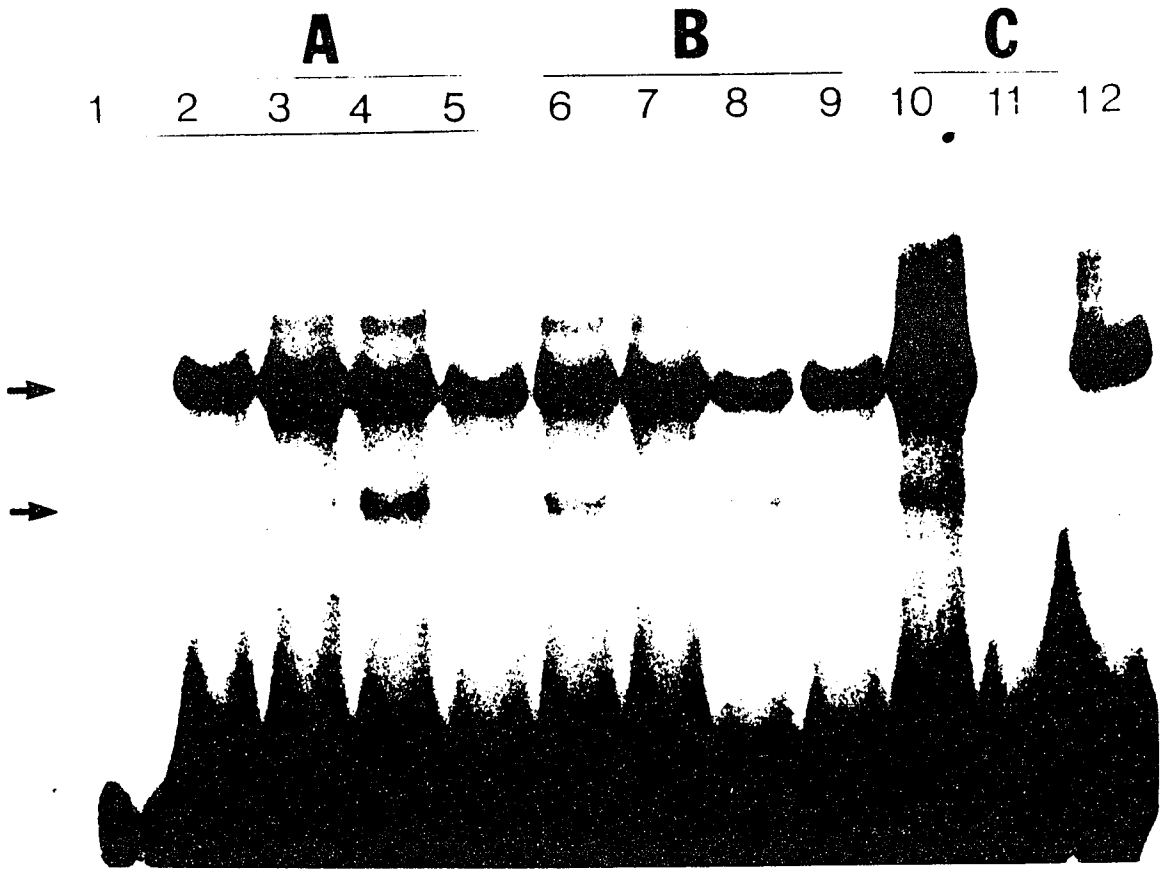


Fig.6

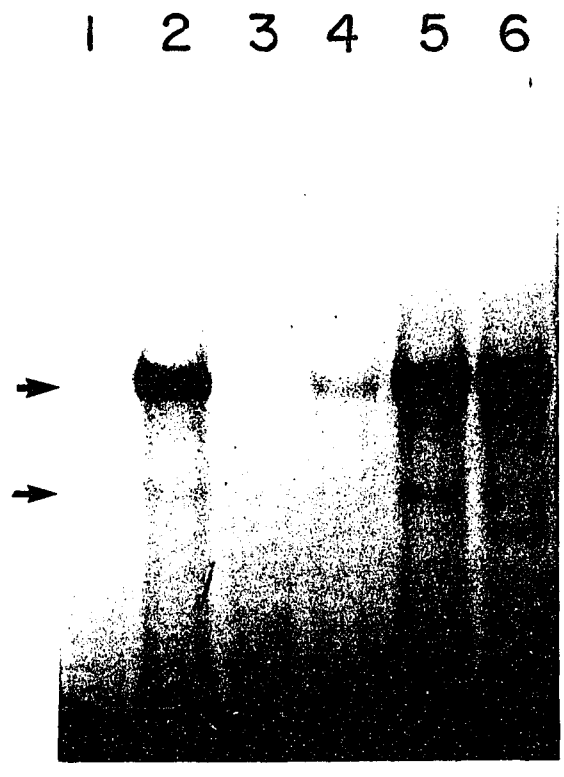
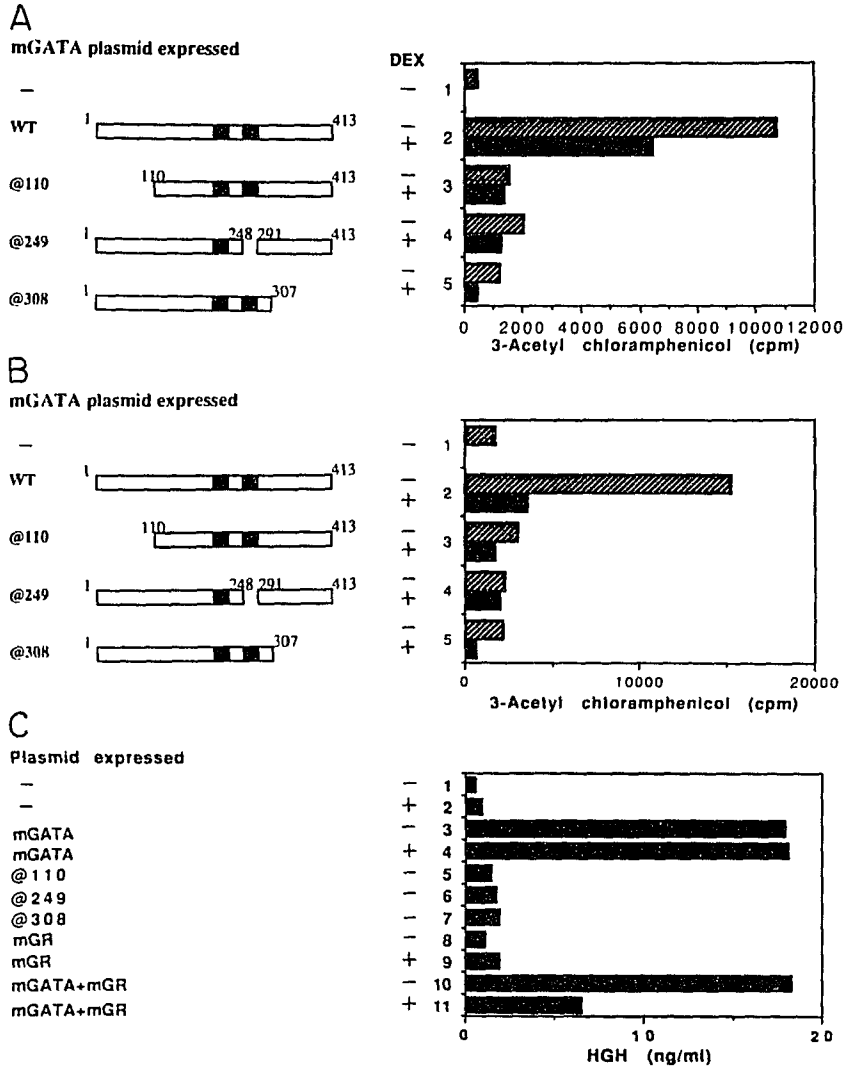


Fig.7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mGATA plasmid expressed
DEX
WT
@110
@249
@308
1
413
110
248 291
307
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3-Acetyl chloramphenicol (cpm)
B
mGATA plasmid expressed
0 10000 20000
3-Acetyl chloramphenicol (cpm)
C
Plasmid expressed
mGATA
mGATA
@110
@249
@308
mGR
mGR
mGATA+mGR
mGATA+mGR
0 10 20
HGH (ng/ml)

Fig.8

D

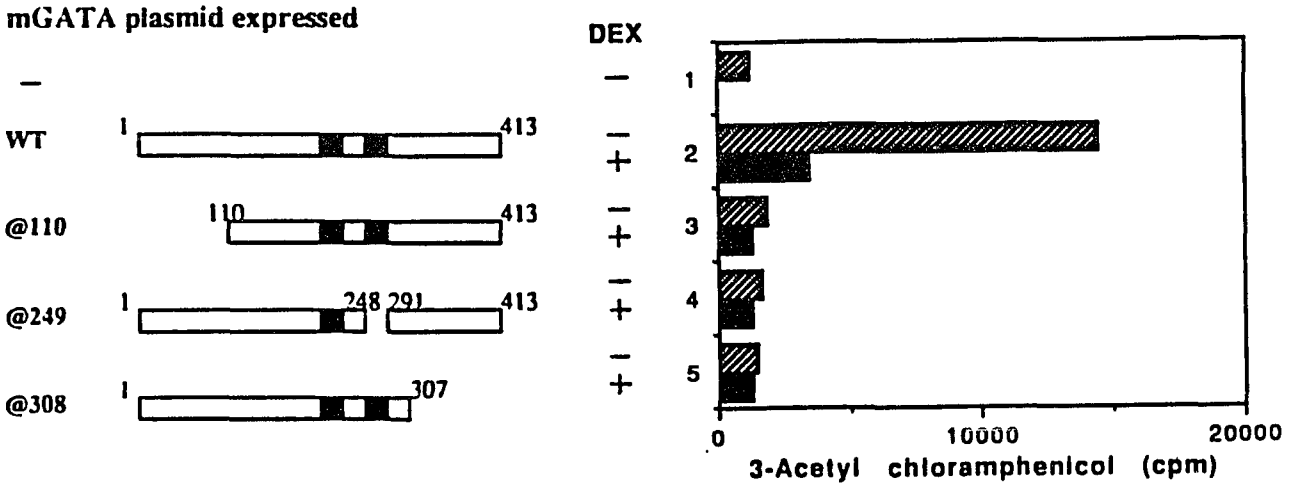
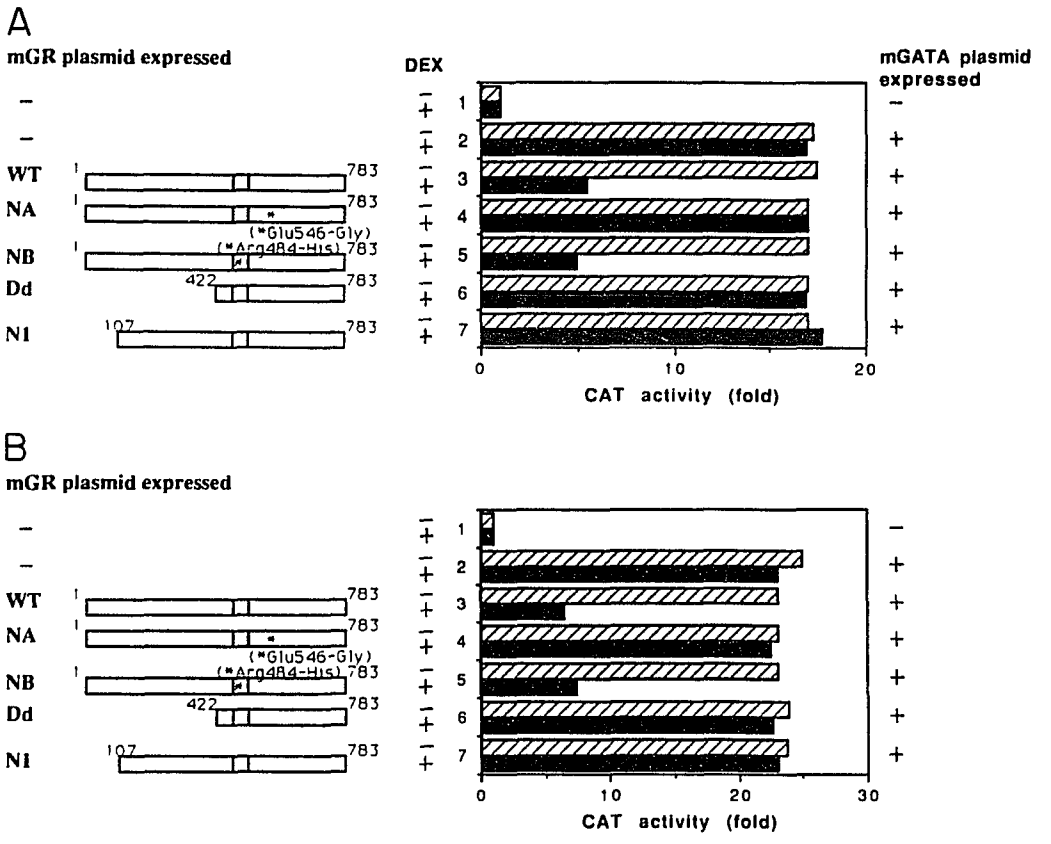

mGATA plasmid expressed
–
WT
1
413
@110
110
413
@249
1
248
291
413
@308
1
307
DEX
–
1
–
+
2
–
+
3
–
+
4
–
+
5
0
10000
20000
3-Acetyl chloramphenicol (cpm)

Fig.8


A
mGR plasmid expressed
DEX
mGATA plasmid expressed
WT
NA
NB
Dd
NI
783
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(*Glu546-Gly)
(*Arg484-His)
CAT activity (fold)
B
mGR plasmid expressed
CAT activity (fold)

Fig. 9

C

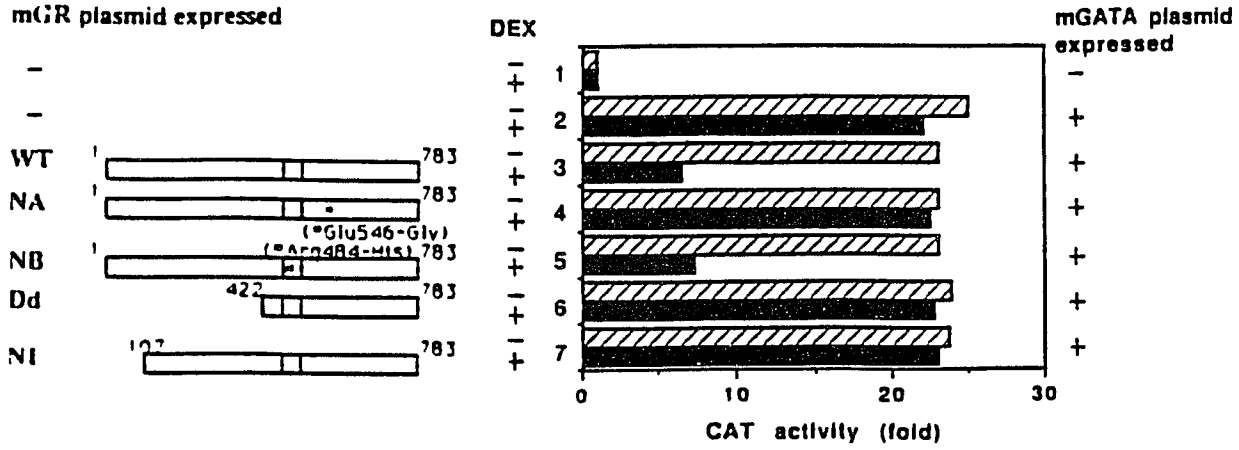
mGR plasmid expressed
DEX
mGATA plasmid expressed
WT
NA
NB
Dd
NI
783
422
(*Glu546-Gly)
(*Arg484-His)
1
2
3
4
5
6
7
0
10
20
30
CAT activity (fold)

Fig.9

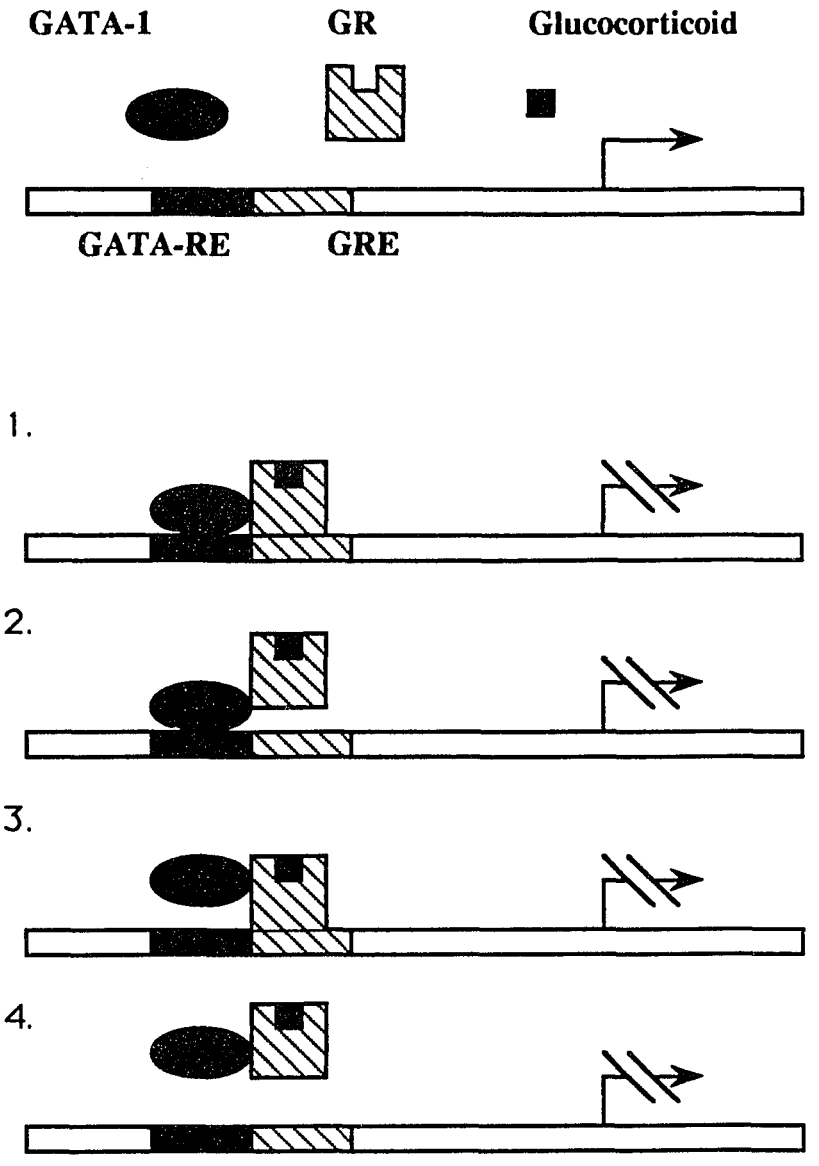

Fig. 10

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