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Frederick Stohlman Jr. Memorial Lecture

Clinical Research Using 3'-Azido-2',3'-Dideoxythymidine (AZT) and Related Dideoxynucleosides in the Therapy of AIDS

S. Broder [1]

Introduction

Pathogenic retroviruses play an etiologic role in the acquired immune deficiency syndrome (AIDS) and its related disorders. While no cure for diseases caused by these agents is available, we are now in an era in which therapy against pathogenic retroviruses is a practical reality. Therapies against the etiologic agent of AIDS [1–3] were made possible by the discovery that a retrovirus, now called the human immunodeficiency virus (HIV), caused the disorder [4–6]. This discovery and the ability to grow the virus in large quantities enabled the development of in vitro techniques to find drugs that inhibit the replication of HIV [7, 8]. Substances that acted against HIV in vitro could then be identified for further research, and the orderly development of drugs thus proceeded. The work was in part a outgrowth of early research on animal retroviral systems in a number of laboratories [9–12]. No one person or group can take full credit for these discovieries, and a great debt is owed to many scientists who pioneered this research. More recently, our group and other groups have observed that certain members of a class of compounds called dideoxynucleosides are potent inhibitors in vitro of the replication of HIV in human T cells [8, 13–26]. In all the compounds, the hydroxy (–OH) group in the 3'-position on the sugar ring is replaced by a hydrogen atom (–H) or another group that cannot form phosphodiester linkages.

3'-Azido-2',3'-dideoxythymidine (also called zidovudine, 3'-azido-3'-deoxythymidine, azidothymidine, or AZT), the first of these compounds to be tested clinically, reduced the morbidity and mortality associated with severe HIV infection [27, 28]. Volberding et al. [29] reported that AZT is effective in delaying progression to fulminant AIDS in asymptomatic patients infected with HIV. Other dideoxynucleosides are now in various stages of clinical testing. Other substances that act at various stages of HIV's replicative cycle also have been shown to block replication in vitro, and some are undergoing clinical testing [7, 31–59]. This article reviews certain clinical applications of one antiretroviral agent, AZT, and discusses the status of several related compounds. It also addresses other approaches to antiretroviral treatment. While an ultimate cure for AIDS will require further basic research, the knowledge already at hand might make a major impact against the death and suffering from this disease in the coming decade. During the decade of the nineties, AIDS is expected to increase, and the disease is likely to become a major cause of death in men, women, and children. In some parts of the world, infant and child mortality could be as much as 30% greater than what one would have expected [60].

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Potential Mechanisms of Action Against HIV

Although there are substantial diffferences among the dideoxynucleosides, we can make several general comments about their mechanisms of action. Each drug likely inhibits reverse transcriptase but must first be activated to a 5′-triphosphate form by various enzymes of a target cell [61–65]. It is the triphosphate form that is active against HIV [61, 66]. The activation process, called anabolic phosphorylation, involves a series of enzymes (kinases) [61–65, 67]. AZT and other dideoxynucleosides, as triphosphates, exert their antiretroviral activity at the level of reverse transcriptase (viral DNA polymerase) [12, 61, 66, 68, 69]. Reverse transcriptase is essential in the replicative cycle of HIV. A great deal is now known about the overall structure of the polymerase domain of HIV reverse transcriptase, about the active site, and about the secondary structure within the active site (e.g., see [69]. When HIV enters a target cell, this enzyme makes a complementary strand DNA copy of the viral genomic RNA and then catalyzes the production of a second, positive strand DNA copy. The genetic information of HIV is thus encoded in a double-stranded form of DNA. Two mechanisms may contribute to the effect of AZT and other dideoxynucleosides on reverse transcriptase. First, as triphosphates, they compete with the cellular deoxynucleoside-5′-triphosphates that are essential substrates for the formation of proviral DNA by reverse transcriptase with inhibition constants generally in the range of 0.005–0.2 μ*M* [61, 70–73]. These concentrations can be attained in cells exposed to the drugs [61–65]. Second, such 5′-triphosphatases act as chain terminators in the synthesis of proviral DNA. Because of the 3′ modification of these compounds, once viral reverse transcriptase adds them to a growing chain of viral DNA, the DNA is elongated by exactly one residue and then terminated [66]. In contrast to HIV reverse transcriptase, mammalian DNA polymerase alpha is relatively resistant to the effects of these drugs (inhibition constants, 100–230 μ*M*) [12, 61, 63, 70], which is one reason for their selective antiretroviral activity in cells that can phosphorylate them. However, mammalian DNA polymerase gamma, found in mitochondria, and DNA polymerase beta are also sensitive to these compounds (inhibition constants, 0.016–0.4 μ*M* and 2.6–70 μ*M*, respectively), and this may be a basis for drug toxicity [12, 61, 63, 70]. It should always be borne in mind that additional mechanisms of activity and toxicity might be at work. It is possible that AZT works through an as yet unidentified intermediate.

AZT and related dideoxynucleosides have activity against certain retroviruses, including HIV type 2, human T-cell lymphotropic virus type I, animal lentiviruses, and murine retroviruses [9–12, 68, 74–78]. (Some congeners also have in vitro activity in an animal model of hepatitis B virus [79] that, although a DNA virus, replicates through an RNA intermediate using a reverse transcriptase-like DNA polymerase [80].) This conservation of activity suggests that the sensitivity of reverse transcriptase to these agents (as triphosphates) is linked to an essential feature of the viral enzyme. Nevertheless, two groups recently reported that certain isolates of HIV from patients who had taken AZT for 6 months or longer had reduced sensitivity to AZT in vitro [81, 82]. Some of the known mutations would be likely to affect the charge or alpha helix content of the catalytic site, or at least the probable catalytic site, of reverse transcriptase. Larder et al. [83] previously showed that the induction in vitro of certain mutations in reverse transcriptase by site-directed mutagenesis could make the enzyme less sensitive to inhibition of AZT triphosphate and phosphonoformate (but also reduce enzymatic activity). However, HIV isolated from patients receiving long-term AZT therapy retains its in vitro sensitivity to inhibition by

most other dideoxynucleosides (such as 2′,3′-dideoxycytidine) and phosphonoformate [82]. Also, preliminary studies suggest that the sensitivity of the reverse transcriptase obtained from these resistant viral isolates to 3′-azido-2′,3′-dideoxythymidine-5′-triphosphate (AZT-TP) did not change [82].

These preliminary findings suggest that while changes in reverse transcriptase may account for the loss of sensitivity to AZT, additional studies are needed to clarify these issues. Many clinical investigators believe that the emergence of AZT-insensitive isolates is a marker of impending clinical progression, but the clinical importance of the reduced viral sensitivity to AZT is not fully known. The potential problem, however, underscores the urgency for additional experimental therapeutic agents and for regimens that employ multiple agents.

Biochemical Pharmacology of AZT

Unlike most nucleosides that enter cells by specialized transport systems, AZT can enter mammalian cells by passive, nonfacilitated diffusion [84]. Once inside, the drug is phosphorylated to a triphosphate form by a series of kinases that usually phosphorylate thymidine [51]. 3′-Azido-2′,3′-dideoxythymidine-5′-monophosphate (AZT-MP) is first produced by thymidine kinase, and two additional phosphates are then added by the sequential action of thymidylate kinase and nucleotide diphosphate kinase to form 3′-azido-2′,3′-dideoxythymidine-5′-triphosphate (AZT-TP), the active moiety [61, 72, 73]. The addition of a second phosphate group to AZT-MP by thymidylate kinase is probably the rate-limiting step for this process [61]. The reaction occurs much more slowly (relative maximal velocity, 0.3%) than the phosphorylation of thymidine-5′-monophosphate, the usual substrate for this enzyme [61], and human cells exposed to AZT accumulate relatively high levels of AZT-MP, but low levels of AZT-TP [61]. AZT-MP binds efficiently to thymidylate kinase, but comes off slowly, thus tying up the enzyme. In certain T-cell lines exposed to very high concentrations of AZT (50–200 μM), decreased phosphorylation of thymidine and decreased levels of thymidine-5′-triphosphate (the normal DNA building block that competes with AZT-TP for reverse transcriptase) have been reported [61, 85]. This may result from the inhibition of thymidylate kinase by AZT-MP (inhibition constant, 8.6 μM) [61]. However, decreased concentrations of thymidine-5′-triphosphate have not been found in other cell lines or with lower concentrations of AZT [85, 86], and whether thymidine kinase inhibition contributes to the bone marrow toxicity induced by AZT is uncertain. Furman et al. [61] originally reported that the levels of deoxycytidine-5′-triphosphate, another building block of DNA, fell in the presence of AZT, but later they concluded that the finding was due to a technical error [87]. It should be stressed that murine cells handle AZT in a very different way from human cells.

Pharmacokinetics of AZT

In culture, AZT inhibits new HIV infection of lymphocytes at levels of 1–5 μM (even under conditions of high multiplicity of infection) [8]. The initial pharmacokinetic studies demonstrated that AZT absorbed well orally (average oral bioavailability, 63%) and that peak plasma levels of 3–4 μM are attained 30–90 min after an oral dose of 200 mg is ingested [27, 88, 89]. The peak plasma concentration is proportional to the amount administered, over a wide range of doses [89]. The serum half-life is only 1.1 h [27, 89, 90]. For this reason, a dosing every 4 h was chosen for phase II testing in patients with advanced disease and then adopted as the recommended schedule. It should be stressed, however, that there is not yet enough information to determine the optimal dose or dosing schedules for AZT. Less frequent dosing

schedules and total daily doses work, but further research is needed. It is likely that total doses of approximately 600 mg per day using an interval of eight hours between doses would be adequate in most adults. The levels of intracellular AZT-TP (the activity moiety of AZT) have a half-life of about 3 h [61]. Thus, effective anti-HIV activity may theoretically be attained with an oral dosing interval of 8 or 12 h. Alternatively, for the optimal therapeutic benefit, it may be necessary to maintain constant plasma drug levels. Resolving these issues will require careful controlled trials.

Approximately 15%–20% of an administered dose of AZT is excreted unchanged in the urine, and 75% is metabolized by hepatic glucuronidation to form 3′-azido-2′,3′-dideoxy-5′-glucuronylthymidine, an apparently inert metabolite that is also excreted into the urine [90]. The enzyme or enzymes responsible for the glucuronidation of AZT may be inhibited by other compounds that share this pathway, and such compounds may prolong the half-life of the drug [91]. In this regard, probenecid inhibits both hepatic glucuronidation and renal excretion and thus reduces the total body clearance of AZT by 65%. Other drugs that undergo hepatic glucuronidation and may, theoretically, inhibit the metabolism of AZT include nonsteroidal anti-inflammatory agents, narcotic analgesics, and sulfonamide antibiotics. Until the interactions of such drugs with AZT have been carefully investigated, clinicians should be aware that they may affect the metabolism of AZT, and AZT may affect theirs. Finally, the metabolism of AZT could be slower in patients with severe hepatic disease. It should be stressed that different nucleoside analogs will show individual pharmacokinetic profiles [27–30, 32, 63, 89–98].

Because HIV can infect cells in the central nervous system and cause dementia, antiretroviral agents used in the treatment of AIDS should be capable of penetrating the brain. Three to four hours after a dose of AZT has been administered, levels in cerebrospinal fluid are approximately 60% of those in plasma (range, 10%–156%) [27, 38, 89, 99], which indicates that AZT can enter the brain by diffusion from the cerebrospinal fluid or possibly through capillaries in the brain. The clinical improvement that occurs in patients with HIV dementia who are given AZT suggests that the drug reaches the site of viral replication in the central nervous system [28, 99]. However, AZT may not cross the blood-brain barrier in all species [100].

Evidence suggests that cells belonging to the monocyte-macrophage series are the most important target cells of HIV infection in the brain [101, 102]. These nonproliferating cells have lower levels of kinases than lymphocytes [103, 104] and dideoxynucleosides may not be efficiently phosphorylated in them. One study indicated that AZT is poorly phosphorylated in peripheral blood monocytes and macrophages and does not protect these cells against infection by the lymphadenopathy-associated virus strain of HIV in vitro [104]. This study is not correct. Two subsequent studies demonstrated that low concentrations of AZT and other dideoxynucleosides profoundly inhibited the replication of a monocytotropic strain of HIV in monocytes and macrophages [105, 106]. These results are consistent with the observation that dementia induced by HIV may be at least temporarily reversed by AZT [21, 99, 107]. The potent activity of AZT in monocytes and macrophages can perhaps be explained by the observation that such cells have very low levels of thymidine-5′-triphosphate, the normal nucleotide that competes with AZT-TP at the level of reverse transcriptase [105]. Thus, the ratio of AZT-TP to thymidine-5′-triphosphate may actually be higher in monocytes than it is in T cells [105]. Additional studies indicate that granulocyte-monocyte colony-stimulating factor, which stimulates the replication of HIV in monocytes [108, 109], increases the entry of AZT into these cells

and potentiates its activity against HIV [109]. However, it is important to use caution in extrapolating these data to clinical applications.

Clinical Application of AZT

In the initial clinical studies of AZT at the National Cancer Institute and Duke University Medical Center, patients with AIDS or AIDS-related complex had immunologic, virologic, and clinical improvement during 6 weeks of therapy [27, 88]. (Three of the 19 patients from the original phase I study, each of whom had AIDS-related complex or Kaposi's sarcoma when they entered the trial, were alive 3.5 years after the initiation of therapy.) Also, several patients with HIV dementia who were given AZT had substantial improvement in their intellectual function, accompanied in some by a normalization in the pattern of use of cerebral glucose (as assessed by positron emission tomography) [99, 100]. On the basis of these results, Wellcome Research Laboratories began a multicenter, randomized, controlled trial of AZT in February 1986 among 282 patients with AIDS (after their first episode of *Pneumocystis carinii* pneumonia) or severe AIDS-related complex [29, 111]. The trial demonstrated a reduced mortality in the patients receiving AZT. By September 1986, 19 patients taking placebo but only one taking the drug had died [28]. After 36 weeks, 39.3% of those taking placebo had died compared with 6.2% of those taking AZT, and after 52 weeks the cumulative mortality in the patients treated with AZT was still only 10.3% (no comparable figure is available for the placebo group, because most were given AZT after September 1986, thus ending the control arm) [28]. Suppressive prophylaxis for *Pneumocystis* pneumonia was not a formal protocol option in the 7-month randomized trial, although a few patients randomly distributed between the two arms received it. Also, 19 of the 144 originally assigned to AZT received more than 6 weeks of such prophylaxis during the 52 weeks after their entry into the study. Eliminating these patients from the analysis does not affect the basis conclusion of the trial. Subsequent studies have suggested that patients with AIDS who receive AZT in conjunction with prophylactic therapy for *Pneumocystis* pneumonia may have a lower mortality than those who receive AZT alone [112]. However, it is important to stress that the precise role of *Pneumocystis* pneumonia prophylaxis in patients who are receiving antiretroviral therapy has not been defined.

The phase II study also showed that patients receiving AZT had a temporary increase in their $CD4^+$ lymphocyte counts (average, $80/mm^3$), fewer opportunistic infections, and an average weight gain of about 0.5 kg, as compared with those receiving placebo [28]. Furthermore, the results confirmed an observation in the phase I study; some patients with cognitive dysfunction induced by HIV improved when given AZT [99, 107]. Finally, patients who received AZT had a decreased viral load as compared with the placebo group in assessments made by measuring levels of serum HIV p 24 antigen [113]. The ability to isolate HIV from cultured lymphocytes, however, was not affected, although there was a delay in the appearance of HIV in cultures [114]. Although serum HIV p 24 antigen levels are an experimental clinical measure of HIV replication, they may be affected by antibodies to p 24 and other factors. Better methods of assessing viral load are urgently needed. The polymerase chain reaction will likely prove useful in this regard [115]. On the basis of this trial's results, AZT was approved for the treatment of severe HIV infection in most countries. In the United States, it was approved in March 1987 for patients who have had *P. carinii* pneumonia or whose CD4+ cell count is below $200/mm^3$. Evidence from the New York State Department of Health indicates that the survival of patients given a diagnosis of AIDS in 1987 increased substantially

over that of patients whose disease was diagnosed in previous years. It is exceedingly likely that the widespread use of AZT contributed to this trend [116]. The dose used in the phase II study is now known to be higher than is necessary for optimal effects.

In both the phase I and phase II trials, it became apparent that the increase in the number of CD4+ lymphocytes induced by AZT may be transient [27, 29, 117], particularly in patients with fulminant AIDS, whose CD4 count often returns to baseline after 16–20 weeks of therapy [28]. In this trend, the contributions of direct drug toxicity, altered host defense mechanisms, and changes in viral sensitivity to AZT are unclear. It is highly probable that no antiretroviral agent will work to maximal advantage if the host immune response is severely damaged. Some patients have had late increases in levels of HIV p 24 antigen even while receiving a constant dose of AZT [118]. In addition, AZT has a number of toxic effects; the most frequent is suppression of bone marrow cells, and anemia is its most frequent manifestation [27, 28, 111, 117]. An increase in the mean corpuscular volume of erythrocytes often occurs before frank anemia [27, 111], but the dose should not be modified on the basis of this measure. Patients receiving AZT may have megaloblastic changes in bone marrow, maturational arrest of erythrocyte lineage, or hypoplastic (rarely aplastic) changes. Hypoplastic changes can occur without an increase in mean corpuscular volume [119, 120]. Bone marrow toxicity occurs more frequently in patients with established AIDS, and in the phase II study, 45% of the patients who had *P. carinii* pneumonia required transfusions or a reduction in dose during the first 6 months of AZT therapy [111]. In a subsequent open trial, only 21% of the patients with AIDS could complete 6 months of full-dose AZT treatment without a reduction in dose or the interruption of therapy [121]. Marrow toxicity is also more frequent in patients with underlying anemia, low CD4+ cell counts, or low (or low-normal) serum folic acid or vitamin B_{12} levels before therapy begins [27, 67, 111]. Pending further study, vitamin-replacement therapy may be useful in patients with low levels of these vitamins. The platelet count is generally spared until late in the course of AZT therapy. In fact, the drug can actually induce increased platelet counts in patients with thrombocytopenia induced by HIV [122, 123].

Other toxic effects of AZT include nausea, vomiting, myalgias, myositis (particularly in patients who receive the drug for more than a year), headaches, abnormalities of liver function, and bluish nail pigmentation [27, 111, 124–126]. Very high doses can cause anxiety, confusion, and tremulousness [27, 117, 127]. These symptoms occasionally develop in patients receiving the current recommended dosage. Finally, a few patients have had seizure, an encephalopathy similar to Wernicke's or Stevens-Johnson syndrome [128–130]. Thus, although AZT decreases morbidity and mortality among patients with severe HIV infection, its use can be associated with substantial toxicity, particularly in those with advanced disease.

Slightly more than 2 years after AZT was first observed to inhibit the replication of HIV in vitro, it was approved by the Food and Drug Administration for the treatment of AIDS. Because of this extraordinarily rapid development, a number of questions regarding its use remain unanswered. In AIDS, as in perhaps no other condition, the line between approved and experimental therapy is difficult to draw.

Physicians frequently ask whether AZT should be administered early in the course of HIV infection. The drug appears to be relatively well tolerated at this stage [131], and recent results of a randomized trial have indicated that HIV-seropositive patients with less than 500 CD4 cells/mm^3 who were given AZT had less frequent progression to severe AIDS-related complex or AIDS than those given placebo [29]. As we learn

more about the factors that make a progression to AIDS highly likely (e.g., high serum HIV p 24 antigen levels or β_2-microglobulinemia) [132, 133], it may be possible to target AZT therapy to patients who are at high risk. At present, we cannot say that starting AZT early in an asymptomatic phase of HIV infection provides a survival advantage over waiting until more symptomatic disease supervienes.

In considering early intervention with AZT, it is of particular concern that the drug may be carcinogenic or mutagenic [134]. Its long-term effects are unknown. Rodents exposed to high doses of AZT for long periods can develop vaginal neoplasms (principally nonmetastasizing squamous cell carcinomas). Male rodents did not develop tumors. The implications of these sex- and site-specific tumorigenic effects in rodents are not clear at this time, but the results provide a warning against complacency when using this class of drug. It is worth emphasizing again that AIDS itself makes the development of certain cancers more likely, and AZT may be associated with the higher incidence of cancers in patients whose immunosurveillance mechanisms are disturbed, simply because it increases their longevity. This has occurred in certain immunodeficiency disorders of childhood, in which advances in the treatment of infections have allowed patients to survive longer [135]. Lymphomas have developed in a significant subset of the original phase I patients between 1 and 3 years after AZT therapy began.

The use of AZT in children with AIDS is an area that is only now being investigated. The high incidence in certain cities of cord blood samples that are seropositive for HIV (for example, 1 in 80 newborns in New York City are seropositive [116, 136]) indicates that the number of children with AIDS will grow in the near future. AIDS is dramatically altering the landscape of obstetrical and pediatric care in many countries. The manifestations of HIV infection in children can differ from those in adults. Neurologic dysfunction and high-grade bacterial infections are much more evident, for example [137], and the patterns of drug toxicity may differ. Ongoing studies at the National Cancer Institute suggest that administration of AZT by continuous intravenous infusion can reverse certain neurologic symptoms associated with HIV in children with AIDS [138]. In some patients, the intelligence quotients returned to what they had been before the disease developed [138]. However, the problems of bone marrow suppression limit that treatment. In both adults and children who have dementia associated with AIDS, considerable bone marrow suppression may be tolerable if antiretroviral therapy can reverse major neurologic deficits. Another unresolved issue is whether AZT can prevent new HIV infection if it is given at the time of viral exposure. Kittens can be protected against feline leukemia virus (a retrovirus) by the administration of AZT at the time of infection [78]. Also, fetal mice can be protected against retroviral infection by the administration of AZT to their mothers [139]. A short course of AZT at the time of exposure (e.g., after a serious needle-stick or laboratory accident) may therefore be useful. However, because it is mutagenic (and carcinogenic in rodents) and can induce chromosomal abnormalities [134], its use in such a setting cannot be recommended except in an approved protocol.

Finally, even in patients for whom AZT is recommended, there is much to learn. The available evidence suggests but does not prove that patients should continue to receive therapeutic doses for as long as they can tolerate the drug. AZT was determined to be effective because it lowered the risk of opportunistic infections and prolonged life. The late decline in the CD4+ cell counts is thus not an indication to stop therapy. However, it is not yet clear how patients who have hematologic toxicity, evidence of clinical progression, or increased levels of serum HIV p 24 antigen while receiving AZT should be managed. Certain factors that

stimulate bone marrow, such as erythropoietin, granulocyte-stimulating factors, or granulocyte-monocyte colony-stimulating factors [109, 110], may reduce the suppression of bone marrow associated with AZT, and these approaches need further research in an academic center.

Biochemical Pharmacology of Other Antiretroviral Dideoxynucleosides Including 2′,3′-Dideoxyinosine (Didanosine)

As noted, a number of dideoxynucleosides other than AZT have antiretroviral activity in vitro [13–26], and several studies of such agents are now enrolling patients. There are substantial differences in the rate at which human cells phosphorylate these compounds and in their enzymatic pathways. These differences are profoundly important to their antiretroviral activities. 2′,3′-Dideoxycytidine (ddC) is, for example much more potent than 2′,3′-dideoxythymidine (ddT) in most human cells because of differences in its phosphorylation [31, 61–65, 67]. Since their rates of phosphorylation differ between species [68], one cannot draw conclusions about their activities in human cells on the basis of their performance in animal cells. 2′,3′-Dideoxyadenosine (ddA), 2′,3′-dideoxyinosine (ddI), and ddC are three compounds with potent activity against HIV in human T cells and monocytes in vitro under study [13, 14, 105] in clinical trials. One of the most active dideoxynucleosides is ddC, a pyrimidine analog [13, 14]. Unlike many cytidine analogs, it is resistant to deamination by the ubiquitous enzyme cytidine deaminase [62, 63]. It is, therefore, stable in plasma and bioavailable after oral administration. After entering a cell, ddC is phosphorylated by a set of enzymes that usually phosphorylate deoxycytidine [62, 63, 141, 142]. Thus, ddC is activated by a different pathway than AZT. Also, ddC does not affect the levels of its competing dideoxynucleoside triphosphate, deoxycytidine-5′-triphosphate [14]. However, its anabolic phosphorylation and activity may be affected by other nucleosides (e.g. thymidine) [143]. ddC is exceptionally potent and the optimal dose to avoid neuropathy is still under study. Total doses of 2 mg per day or less are being tested in adults.

It is worth emphasizing that this entire class of nucleoside analogs represents a new area of clinical research. These drugs have antiretroviral activity; however, they also have considerable potential for side effects. These drugs should be administered only by physicians who are well versed in their properties.

ddA and its immediate metabolite ddI are purine analogs with in vitro activity against HIV [13] and, unlike AZT, relatively little toxicity against bone marrow precursor cells [144]. Within cells, ddA can be phosphorylated to its active 5′-triphosphate moiety [64]. It is also susceptible to deamination by adenosine deaminase and forms ddI [64]. In human plasma and cell extracts, this conversion occurs almost instantaneously [26, 64]. As mentioned, ddI has potent in vitro activity against HIV [13] because it can be metabolized in human cells to form ddA-5′-triphosphate through a complex series of reactions [65]. Interestingly, ddI uses the enzyme 5′-nucleotidase to undergo the initial phosphorylation it needs for activation and ultimate salvage back to ddATP. Thus, for many purposes the two drugs can be considered identical. Once ddA and ddI are converted to ddA-5′-triphosphate in cells, they remain there for a relatively long time – their intracellular half-life is more than 12 h [145]. Thus, even with their short plasma half-life, they may be clinically effective when administered relatively infrequently (e.g., every 8–24 h). Unlike AZT or ddC, ddA and ddI undergo solvolysis (cleavage) in acid reactions to form a purine base and dideoxyribose [64]. This may lower their capacity for oral absorption, and they must be used with antacids or buffers. High concentrations of the free purine base of ddA, adenine, have been reported

to cause renal damage [146]. The free base of ddI, hypoxanthine, does not have similar toxicity, and it may, therefore, be preferable for oral administration. Preliminary results from a phase I trial and ddI suggest that it is an active antiretroviral drug [9]. Some patients have now received this drug for more than 2 years. The major side effects to data have been a reversible peripheral neuropathy and acute pancreatitis. In some cases, the pancreatitis may be lethal. It appears that a prior history of pancreatic disease is risk factor for this complication. Significant diarrhea and hypokalemia may occur. These side effects seem to be dose related. Patients who have advanced disease or who are debilitated have an increased risk for toxicity. At doses less than 8 mg/kg per day, serious side effects are significantly less common than at higher doses. In an average adult, total doses should not exceed 500 mg per day. It is possible that even lower doses eventually will be found active. The use of alcohol is contraindicated in patients receiving ddI due to the possibility of pancreatitis.

Certain analogs of ddA (e.g., 2′,3′-dideoxy-2′-fluoro-ara-adenosine) are resistant to acid hydrolysis [21] and, therefore, may have better bioavailability than ddA. Also, certain 2-halogen-substituted forms of ddA are resistant to deamination [26], and they may be directly phosphorylated and not follow the ddI pathway. Whether clinical studies with such forms will produce compounds superior to ddA or ddI is not known; ddI has potent in vitro activity against HIV in its own right [13], and the issue of acid instability can be addressed by simple measures such as the buffering of gastric secretion.

Like AZT, a number of analogs of ddT have been tested for activity against HIV in vitro. Many were inactive, but a few blocked the replication of HIV in human T cells [14, 16, 17, 22, 147]. An unsaturated form of ddT called 2′,3′-didehydro-2′,3′-dideoxythymidine is about as active as AZT on a molar basis [16, 17]. Unlike AZT, it does not affect the activity of thymidylate kinase [148], whether it induces bone marrow toxicity remains undetermined. The dose-limiting toxicity is peripheral neuropathy. Finally, a 3′-substituted uridine analog, 3′-azido-2′,3′-deoxyuridine, which appears to be activated by the same enzymes that phosphorylate AZT, has some anti-HIV activity in vitro [19, 149]. All these dideoxynucleosides have an intact oxacyclopentane (sugar) ring. However, several acyclic compounds (adenallene, cytallene, and a phosphonyl-methylethyl purine derivative) also have activity against HIV in vitro as single agents. Such compounds provide new relation between structure and activity and may be of value in developing a new class of anti-HIV agents.

Clinical Research with Dideoxycytidine

Although AZT can prolong the lives of patients with AIDS, there are some limitations to its use. The hematologic toxicity of AZT is not inextricably linked to its antiviral effect, and we can expect that other agents will be worth exploring or have different patterns of toxicity. In vitro testing and studies of animal toxicology can provide clues as to which drugs are likely to have favorable therapeutic results. Ultimately, however, the issues can be resolved only by testing in patients, and an effort is now under way to test several of these agents in patients with AIDS or related conditions. The first to be studied clinically (after AZT) was ddC, which has potent activity against HIV in vitro at concentrations of 0.01–0.5 μM, depending on the viral dose used in the assay system [13, 69, 70]. It is well absorbed when given orally, and peak levels of 0.1–0.2 μM can be attained after the oral administration of 0.03 mg/kg body weight [30]. Like AZT, ddC has a half-life of slightly more than 1 h. It differs from AZT in that it is excreted by the kidneys [30, 150]. Finally, ddC penetrates at least partially the cerebrospinal fluid [30, 150].

Both the initial study of ddC [30] and a subsequent trial [151] found evidence of clinical activity against HIV. Nearly all the patients who received daily doses of between 0.06 and 0.54 mg/kg had decreased levels of serum HIV p24 antigen [30, 151], and most had small increases in the number of CD4+ cells by week 2 [30]. Furthermore, some had an increase in antigen-induced T-cell proliferation in vitro [30]. The decrease in levels of p24 antigen persisted in some patients for at least several weeks after the drug was withdrawn. In others, however, the immunologic and virologic values moved toward baseline after several weeks despite the continued administration of ddC [30]. One purpose of these studies was to define the dose-limiting toxic effects of ddC. In a number of patients, particularly those receiving higher doses, maculovesicular cutaneous eruptions, aphthous oral ulcerations, fever, and malaise developed after 1–4 weeks of therapy [30, 151, 152]. These symptoms usually resolved in 1–2 weeks even with continued therapy. However, after several months of continuous therapy with daily doses of 0.06 mg/kg or more, most patients had a painful sensory motor peripheral neuropathy (involving mainly the feet) that became the dose-limiting toxic effect [30, 151]. This neuropathy appeared earlier, was more severe, and lasted longer when the highest doses were tested; some patients receiving the highest doses still had persistent, moderate sensory loss and pain a year after the drug was discontinued [151, 153]. Neurotoxicity resolved much more quickly, however, in patients receiving lower doses [30, 151, 153]. One metabolic product of ddC in human cells is dideoxycytidine diphosphate choline [62], which could conceivably contribute to the neuropathy. Alternatively, the neuropathy may result from an inhibitory effect of ddC-5′-triphosphate on mitochondrial DNA polymerase gamma (inhibition constant, 0.16 μM) [63, 154]. Thus, a search for ddC congeners which would not affect mitochondrial DNA synthesis is under way. Scientists at Hoffmann-La Roche have begun studying a fluorinated version of ddC which may spare mitochondrial DNA polymerase.

A continuation of the study of Merigan et al. [151] and a separate study organized by M. Gottlieb and W. Soo (personal communication) have shown that many patients can tolerate lower doses of ddC (0.03 mg/kg per day) for 6 months or more; mild, readily reversible neuropathy developed in a minority of patients. This dose of ddC was associated with a decline in HIV p24 antigen levels and an increase in the number of CD4+ lymphocytes in most patients. Since the toxicity of ddC is strikingly different from that of AZT, combining the two agents may reduce overall toxicity. To test this approach, a group of patients with AIDS or AIDS-related complex followed a regimen alternating AZT (200 mg every 4 h) and ddC (0.09 or 0.18 mg/kg per day) therapy in 7-day periods [30, 155]. It was hoped that neuropathy would not occur or would occur later with the intermittent administration of ddC. Preliminary results suggest that the toxicity of both agents can be significantly reduced. Some patients have now tolerated the regimen for more than 36 months [155] (unpublished data). Overall, the patients had an average increase of more than 70 CD4+ cells/mm^3 at week 22, sustained decreases in serum p24 antigen levels, and a mean weight gain of 5 kg (not caused by fluid retention) [30, 155]. It is interesting to note that on low-dose or intermittent dosing regimens, once patients pass the 6-month mark without neuropathy, they may have a significant probability of avoiding serious neuropathy on continued administration of ddC. Next to AZT, ddC has been given to patients longer than any other dideoxynucleoside. It is probable that ddC will find its best use as part of a combination regimen with AZT.

Other Anti-HIV Agents in Preclinical and Clinical Development

This article has focused on the use of dideoxynucleosides as antiviral agents, in part because they are bioavailable after oral administration and because data from several studies support their virustatic activity in vitro. This is, however, by no means the only approach being investigated for the treatment of AIDS. The genome and replicative cycle of HIV are very complex, and several stages of replication may, therefore, be potential targets for antiretroviral therapy [31, 67, 156, 157]. Already, a number of agents that may act at various stages have been defined. Although an extensive review of these other approaches is beyond the scope of this article, a few points are worth stressing. Certain agents under study appear to act by inhibiting the initial binding of HIV to its CD4 glycoprotein receptor on target cells [36–38, 41, 42, 52–56, 158–160]. Using molecular biologic techniques, several groups recently reported truncated soluble forms of CD4 that lack the transmembrane and cytoplasmic domains [52–56]. At concentrations of 2–20 μg/ml, these forms inhibited the binding of HIV to T cells, the formation of syncytia, and the infection of T cells [52–56]. A potential advantage of this approach is that soluble CD4 is likely to inhibit, to some degree, all forms of HIV that use CD4 as the cell receptor. Also, agents that act at the cell surface may block cell death induced by syncytia, which can occur even when the target cell is not infected by HIV [38, 161, 162]. Phase I trials of recombinant CD4 are now under way. Second-generation versions of CD4 (such as CD4-immunoglobulin hybrid proteins) retain their activity against HIV in vitro, but may gain other desirable properties, such as a longer circulating half-life [163]. A phase I trial of such CD4-immunoglobulin hybrids is now under way at the National Cancer Institute and at other academic centers. Also, forms of recombinant CD4 linked to *Pseudomonas* endotoxin or ricin selectivity kill cells expressing HIV envelope proteins in vitro [164, 165]. In patients, such agents might selectively kill cells that can replicate HIV without being killed by the virus (e.g., macrophages). Unfortunately, CD4 does not necessarily bind to the gp120 of primary isolates (as opposed to laboratory isolates) with high affinity. This may mean that very high doses of CD4 need to be used.

Recently, low-molecular-weight dextran sulfate (7000–8000) was found to inhibit the infectivity of T cells by HIV [36–38]. This polyanionic polysaccharide also appears to inhibit the initial binding step [38]. A phase I/II trial of orally administered dextran sulfate suggested that it had little toxicity but also little effect on the number of CD4+ cells or serum p24 antigen levels [166]. However, dextran sulfate has since been found to be very poorly absorbed when given orally, and studies of intravenous dextran sulfate are needed in order to assess this agent. Other molecules in this class are worth studying by parenteral administration.

Recent advances in our understanding of the biochemistry of HIV replication have made the testing of new approaches to therapy possible. For example, antisense phosphorothioate oligodeoxynucleotides, which can bind to specific segments of the HIV genome, have sequence-specific inhibitory effects that may result from the arrest of translation after its hybridization to messenger RNA [48]. Interestingly, such compounds may also inhibit the replication of HIV in a manner that is not sequence specific [47]. Alteration of the sugar moiety of viral glycoproteins (e.g., by inhibitors of trimming glucosidases) reduces the infectivity of the resulting viruses [57, 58]. In addition to dideoxynucleosides, other agents may act at the level of reverse transcriptase. In particular, phosphonoformate, a pyrophosphate analog with activity against herpesvirus, has activity against HIV in vitro. Several pilot trials suggest that this drug can reduce serum HIV p24 antigen levels in patients with

HIV infection [167, 168]. However, no reliable oral formulation is available, and this remains one drawback of this drug.

There is a growing interest in developing drugs that inhibit the protease of HIV. During the next few years, it is likely that several protease inhibitors will enter clinical trials.

Several agents that act at different stages of viral replication (e.g., interferon-α) have synergy with AZT in vitro [34, 36, 40] and this could theoretically result in better treatment in patients. Interferon-α may be particularly interesting in this regard, because it also has a direct antitumor effect against cutaneous Kaposi's sarcoma [169–172]. In a similar vein, the antiherpes drug acyclovir, which has little activity against HIV alone, can potentiate the anti-HIV activity of AZT in vitro [14, 31]. In a pilot clinical trial, patients with AIDS or AIDS-related complex tolerated these drugs together for 10–30 weeks [32]. A theoretical advantage of the regimen is that suppressing the replication of herpesvirus may secondarily reduce the replication of HIV since a product of herpesvirus, ICPO (infected-cell protein), can increase the initiation of HIV transcription [173, 174]. The possible suppression of human herpesvirus 6 (human B-cell lymphotropic virus), which can infect lymphoid cells [175], may also be relevant. (In a related fashion, adenovirus enzyme-immunoassay product can also amplify HIV transcription [173]. Certain dideoxynucleosides can inhibit the replication of adenovirus [176] and thus may conceivably reduce the replication of HIV.) Acyclovir has been reported to be at least additive with AZT in inhibiting the replication of Epstein-Barr virus, and it could theoretically benefit patients infected with both that virus and HIV [177–179]. Whether AZT and acyclovir together offer a therapeutic advantage over AZT alone is not yet clear. Only properly controlled clinical trials can answer this point.

Not all combinations of anti-HIV drugs have synergistic or even additive effects. For example, the nucleoside analog ribavirin inhibits the phosphorylation of AZT in vitro and blocks its activity against HIV [50]. Ribavirin, however, increases the phosphorylation of purine analogs such as ddA through complex mechanisms involving its ability to inhibit inosine monophosphate dehydrogenase [180]. Ribavirin can be given orally and may in theory potentiate the anti-HIV effects of ddA or ddI. Unfortunately, one cannot predict from first principles whether this kind of potentiation would be good or bad. Once again, only carefully controlled clinical trials can resolve this issue. These in vitro observations should alert clinicians to the possibility of unexpected interactions among agents, and they are an argument against ad hoc experimentation with antiretroviral therapies outside approved clinical trials.

Conclusion

In this article a number of new therapeutic agents and strategies have been discussed. We now have at hand a number of approaches that can inhibit the replication of HIV in vitro. These approaches, as well as a number of additional developments which are in the offing, can be expected to induce clinical improvement and prolong life even in patients with advanced AIDS. The progress against the mortality caused by AIDS is noteworthy in its own right, but there have been a number of advances that have improved the quality of life. For example, the incidence of dementias ascribable to AIDS has been noted to have decreased after the introduction of AZT [181].

HIV infection is probably a lifelong process. It now appears highly likely that a complete latency phase does not exist. Rather, many, if not all patients, have circulating infectious HIV particles present in their plasma even when the disease is clinically quiescent. Thus, it is perhaps unrealistic to expect a single drug to provide therapy for all patients. The experiences with cancer therapy, as well

as the experiences with other serious infections, suggest that a combination of drugs will produce superior clinical outcome and less toxicity than any single therapy used alone [182]. Combination therapy may also delay or prevent the emergence of viral resistance. Just as in the treatment of certain leukemias or advanced bacterial diseases, optimal therapy against HIV may require at least three different phases: induction, consolidation, and maintenance. It is worth noting that the drugs and biological agents, as well as the relevant doses of such drugs and agents, may vary in each phase. At present, the only formally approved antiretroviral agents are AZT and ddI. AZT has been proven to reduce morbidity and mortality above and beyond any effect of aerosolized pentamidine in severe cases of AIDS [183]. Nevertheless, several virustatic drugs in the same general family are being tested in patients, and it seems highly probable that AZT is not the only agent which eventually will prove effective against HIV. As with a number of other therapies used in life-threatening disorders, AZT may have a relatively low therapeutic index in some patients. Therefore, it is very important that clinicians pay close attention to its clinical pharmacology and to the specific patient responses that occur following initial therapy. As new experimental agents are tested and become more widely available, it is important that careful adherence to the principles of clinical trials be a major priority if we are to succeed in the mission of developing better therapeutic options. As simpler assays to measure plasma drug levels become available [184, 185], their results conceivably may provide useful data in the optimal management of HIV infections. A number of studies are now under way to test whether various agents should be administered to patients with early HIV infections and to explore other therapeutic regimens. In the coming decade, it seems highly probable that major advances will occur against the death and suffering caused by HIV, but this progress can be ensured only if the principles of scientific drug development and controlled trials are maintained.

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Frederick Stohlman Jr. Memorial Lecture

The Human β-Globin Locus Control Region*

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Introduction

The human β-globin gene cluster spans a region of 70 kilobases (kb) containing five developmentally regulated genes in the order 5′-ε,$\gamma_G\gamma_A$,δ,β-3′ (Fig. 1). The haematopoietic tissue in the early stages of human development is the embryonic yolk sac and the ε-globin gene is expressed. This is switched to the γ-globin genes in the foetal liver and the δ- and β-globin genes in adult bone marrow (Fig. 2; for review, see [9]). High levels of these genes are expressed in circulating red blood cells (RBC), giving rise to 90% of the total soluble protein. RBC are derived from a pluripotent stem cell which can differentiate along alternate pathways to erythrocytes, platelets, granulocytes, macrophages and lymphocytes. During the transition to erythroblasts which have lost the capacity to proliferate, the β-globin genes become transcriptionally activated achieving messenger RNA (mRNA) levels of more than 25000 copies per cell.

A large number of structural defects have been documented in the β-globin gene locus (for review, see [9, 44]). These defects are responsible for a heterogeneous group of genetic diseases collectively known as the β-thalassaemias, which are classified into β, δβ, γδβ, etc. thalassaemia subgroups according to the type of gene affected. In a related condition, hereditary persistence of foetal haemoglobin (HPFH), γ-globin gene expression and hence HbF (fetal haemoglobin) production persist into adult life. These clinically important diseases provide natural models for the study of the regulation of globin gene regulation during development. Most interesting in terms of transcription are the promoter mutations and deletions. The δβ-thalassaemias and a number of the HPFHs are associated with an elevated expression of the γ-genes in adult life as a result of deletions of varying size. Analysis of these deletions has suggested that they act over considerable distances, to influence differential gene expression within the human β-globin domain.

The Locus Control Region

The existance of a region that activates the entire β-globin gene cluster first became apparent from the study of a heterozygous γβ-thalassaemia (Fig. 1) [31]. This patient contained one deletion allele which lacked 100 kb, eliminating the entire upstream region but not the β-globin gene [57], which was shown to be completely normal [31, 64]. The other allele was expressed in the patient and it was therefore not a lack of *trans*-acting factors which silenced the mutant chromosome but an important control region had be missing. A set of developmentally stable, hypersensitive sites, 5′ HS1, 2, 3 and 4, were shown to be present upstream in the deleted region, and these

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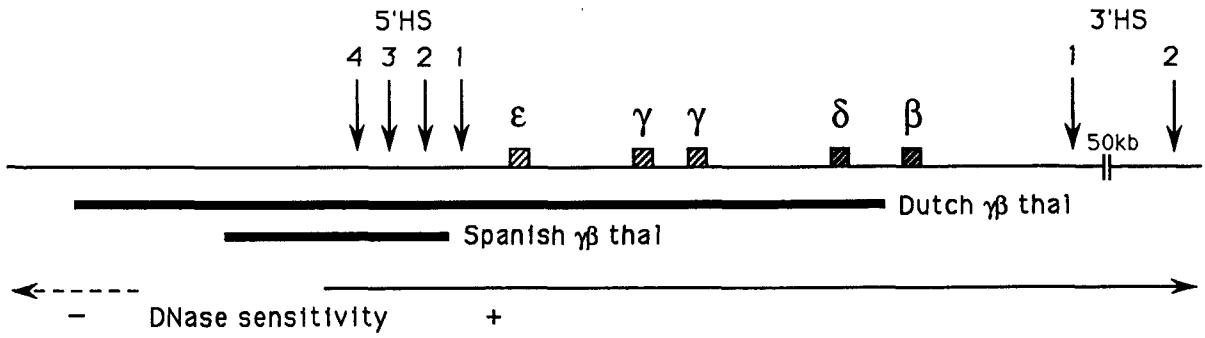

Fig. 1. Schematic representation of the β-globin locus. *Boxes* indicate the different genes which are all transcribed from left to right. The *vertical arrows* indicate DNase hypersensitive sites. The *four arrows* mark the LCR containing 5'HS1, 2, 3 and 4 upstream of the ε-gene. The *two arrows* downstream of the β-gene are 3'HS1 and 2. The *black bars* represent two in vivo deletions which eliminate the function of the LCR. The *horizontal arrows* indicate low (−) and high (+) sensitivity of the chromatin to DNaseI digestion

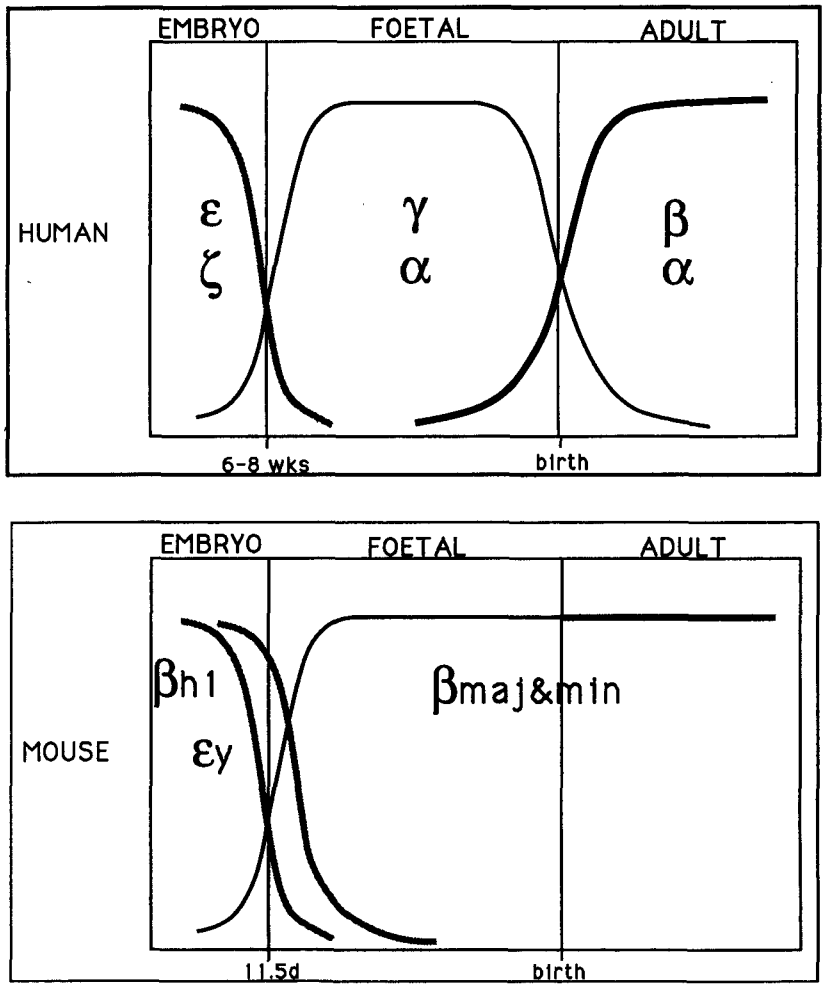

Fig. 2. Schematic representation of the developmental expression patterns of globin genes in human and mouse. The curves refer to the β-like genes only

were potential candidates for such a locus control region (LCR; Fig. 1) [17, 27, 60]. Linkage of this region to a cloned β-globin gene resulted in erythroid-specific high-level expression of the gene in transgenic mice and in tissue culture cells. This expression is dependent on the copy number of the transgene and independent of the integration site in the host genome [3, 27], a phenomenon which had not previously been observed in transgene expression. This posed two questions: How is independence of the site of integration achieved? And how is the LCR involved in globin gene switching?

Position independence and copy number dependence can theoretically be explained by at least two independent mechanisms; either positive activation by the LCR is always achieved, overriding position effects that could be present, or (and) the region contains a locus border element(s) (LBE) that insulate it from neighbouring regions. Matrix attachment sites (MAR) [22, 30] or "A" elements [4, 53] could be LBEs, and we initially speculated these were part of the LCR in addition to activating sequences [27]. However, preliminary experiments indicate that this is not the case and that such a border may be located further upstream. The latter is based on the fact that the DNaseI sensitivity of chromatin is strongly decreased in the sequences 25–30 kb 5′ to the LCR (Fig. 1) [19, 31, 57]. At least 150 kb of chromatin in the 3′ direction is sensitive under the control of the LCR [19], suggesting that such sequences are not present for a considerable distance 3′ (Fig. 1). The position independence we observe is therefore due to a dominant activation of transcription by the LCR, perhaps by creating very stable interactions between the LCR and the genes. Consequently, positive position effects would only be present in the background and only become apparent in situations where the linked gene is suppressed [12] (see below for discussion). Position effects are not observed at low levels of expression when part of the LCR or mutations in the LCR are used. This indicates that the interaction between the LCR and the promoter is dominant except when the promoter is suppressed [10, 12, 18, 20, 49, 54]. In agreement with the deletion observed in a Hispanic γβ-thalassaemia (Fig. 1) [13], the main activity of the LCR is associated with HS2, 3 and 4 [10, 18, 20, 54, 61], which can each activate a linked transgene, independent of the site of integration. A number of protein binding sites have been mapped to these fragments, in particular, sites for two erythroid-specific factors and several ubiquitously expressed factors. A number of the binding sites are present in all three active sites (Fig. 3) [*43*, *45*, *55*]. One of the shared factors is the erythroid/megakaryocyte-specific factor GATA-1 [38, 48], which has been shown to be essential for erythroid development [42]. Deletion of GATA-1 binding sites prevents erythroid-specific induction of the β-globin gene [11], and the protein has been shown to have transcriptional activation properties [38]. However, the presence of GATA-1 binding sites per se is insufficient to give position-independent expression, e.g. the flanking regions of the human β-globin gene contain at least six GATA-1 binding sites [11, 63], but do not confer integration site-independent expression [32, 34, 58]. However, all three active 5′ HS contain two closely spaced GATA-1 sites in opposite orientations. This arrangement is also observed in the chicken β-globin enhancer, which appears to provide position-independent expression [47]. Possibly an inverted double GATA-1 site is a key component in erythroid-specific, position-independent activation and GATA-1 can interact with itself or one of the other GATA proteins to achieve this [66]. Classical enhancer activity is only associated with 5′HS2 [41, 61], and not with the others. Dissection of the HS2 showed that a number of proteins are bound to the core fragment (Fig. 3) [55]. Attention has been focused on a double consensus sequence for the *jun/fos* family of DNA binding proteins which appeared crucial for HS2 activity [41, 52, 55]. Several pieces of evidence

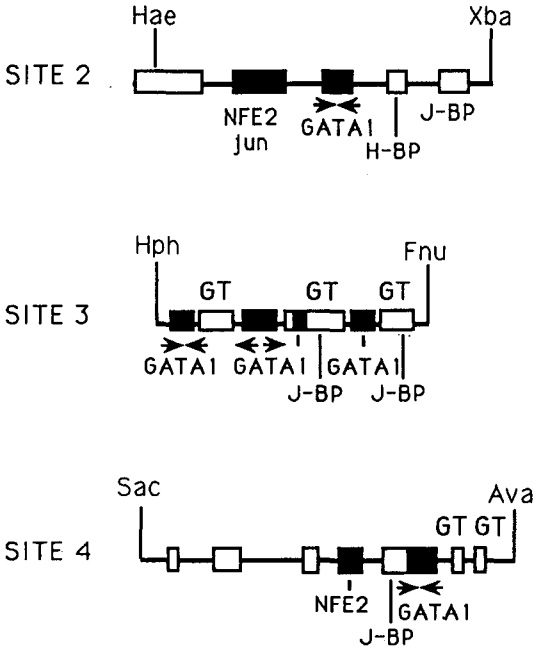

Fig. 3. Summary of factor binding sites to the minimal fragment of 5'HS2, 3 and 4, which provide position-independent expression in transgenic mice. Individual factors are described in the text. *Black boxes* indicate erythroid-specific factors; *open boxes* indicate ubiquitous factors. *GT* indicates a GT-rich motif [43]

show that the functional activator which interacts with the *jun/fos* binding site is NF-E2, originally described as interacting with another erythroid-specific gene, that for porphobilinogen deaminase [39, 40]. Two NF-E2 molecules and at least one other protein binding at two non-equivalent sites are involved [56]. However, the presence of this double NF-E2 sequence alone is insufficient to provide high levels of expression [55] and when the *jun/fos* binding site is removed from the 300 bp core fragment, HS2 retains the ability to activate a linked β-globin gene in a copy number dependent fashion, albeit at low levels (Fig. 4) [56]. We therefore conclude that the 5'HS2 NF-E2 region has strong enhancer activity but that it is not necessarily required to obtain position-independent globin gene activation.

All the other factors which have been shown to interact with LCR sequences, including the factors H-BP and J-BP, are ubiquitous proteins [56]. This suggests that a combination of erythroid-specific and ubiquitous factors may be required to render the β-globin gene independent of its site of integration. The (abundant) ubiquitous factors shared by the three HS of the LCR which have been studied to date are Sp1 and TEF-2 [23, 65], but a simple multimerized combination of a GATA-1 and a Sp1/TEF-2 binding site is not functional (S. Philipsen, unpublished results). We therefore think that other, as yet less well characterised factors may be involved in LCR function.

The LCR and Disease

The discovery, characterization and mapping of the LCR has enabled the pursuit of two novel approaches to the study of globin-related diseases. Firstly it allows high-level expression of disease genes such as the β^s gene which is responsible for sickle cell disease. By linking this gene in combination with human α-globin genes several laboratories have succeeded in producing transgenic mice which show sickle cell disease [26, 50, 59]. High levels of human haemoglobin S can be obtained in mice and the RBC of these mice show a pronounced change in shape when deoxygenated (Fig. 5). We are presently improving this model for two reasons, firstly, to study the effects of sickle cell disease on the progression of infection by different malaria strains, and secondly, to be able to study the progression of sickle cell disease and the treatment thereof by new protocols, in particular the development of gene therapy. The latter has been given new hope by the mapping of the minimal elements that give the full activity of the LCR. The LCR can now be incorporated into retroviral vectors to develop therapy protocols and preliminary experiments (F. Meyer, personal communication) indicate that the LCR will provide high levels of expression in this context in mice.

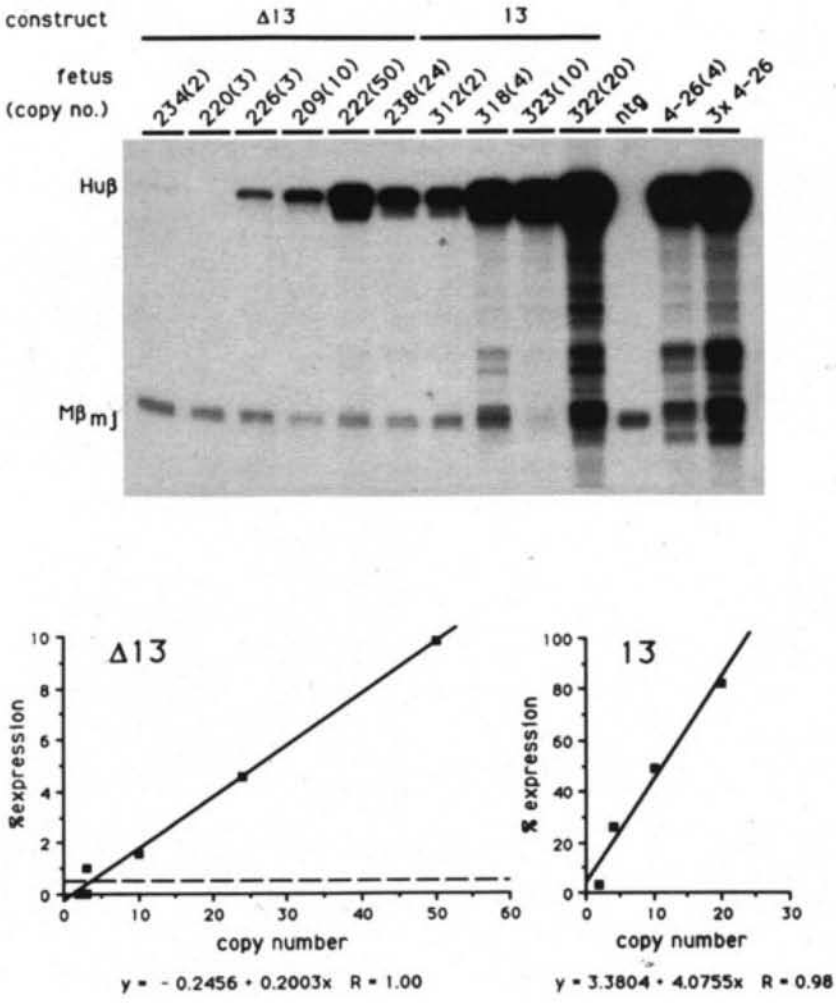

Fig. 4. S1 nuclease analysis of HS2 constructs containing the NF-E2 sites (*13*) or not (*Δ13*) in transgenic mice. Foetal liver RNA (day 13.5) was assayed using a mixed probe S1 nuclease experiment using the 5′ human β-globin probe and the mouse βmaj probe [56]. Specific activities were 10:1 for Hu β:M β. Protected products are indicated on the *left*. The 200 series of transgenic mice contains the Δ13 construct and the 300 series contains the 13 construct. Copy numbers are shown in *parentheses*. *Lower pnael* depicts a quantitation experiment of the S1 protection analysis using the Huβ5′ probe and a mouse α-globin probe as an internal control. The % expression is given as the total Huβ-globin signal divided by the total mouse α-globin signal (adjusted for specific activities). This was plotted against the copy number. The *line* represents the result of a linear regression analysis on the data points. The *R* value, the correlation coefficient, indicates very high correlation with a straight line ($R = 1$). The *dashed line* in the Δ13 graph represents the minimal level that can be measured accurately

Developmental Regulation of the β-Globin Locus

Genetics. The study of globin gene switching has been assisted by the characterization of deletions and point mutations which affect expression of the γ and β-genes. Point mutations in the γ-promoters have been linked to HPFH phenotypes and these can be divided into two groups (Fig. 6).

Those clustered around the distal CAAT box appear to result in the loss of factor binding sites [21, 36], suggesting that this region may contain a binding site for a negative regulator. For example, a 13-bp deletion which removes the distal CAAT box results in a very strong HPFH (60%) [25]. Interestingly, a recently described Japanese HPFH (20%) is associated with a point mutation in the CAAT sequence of the distal CAAT box

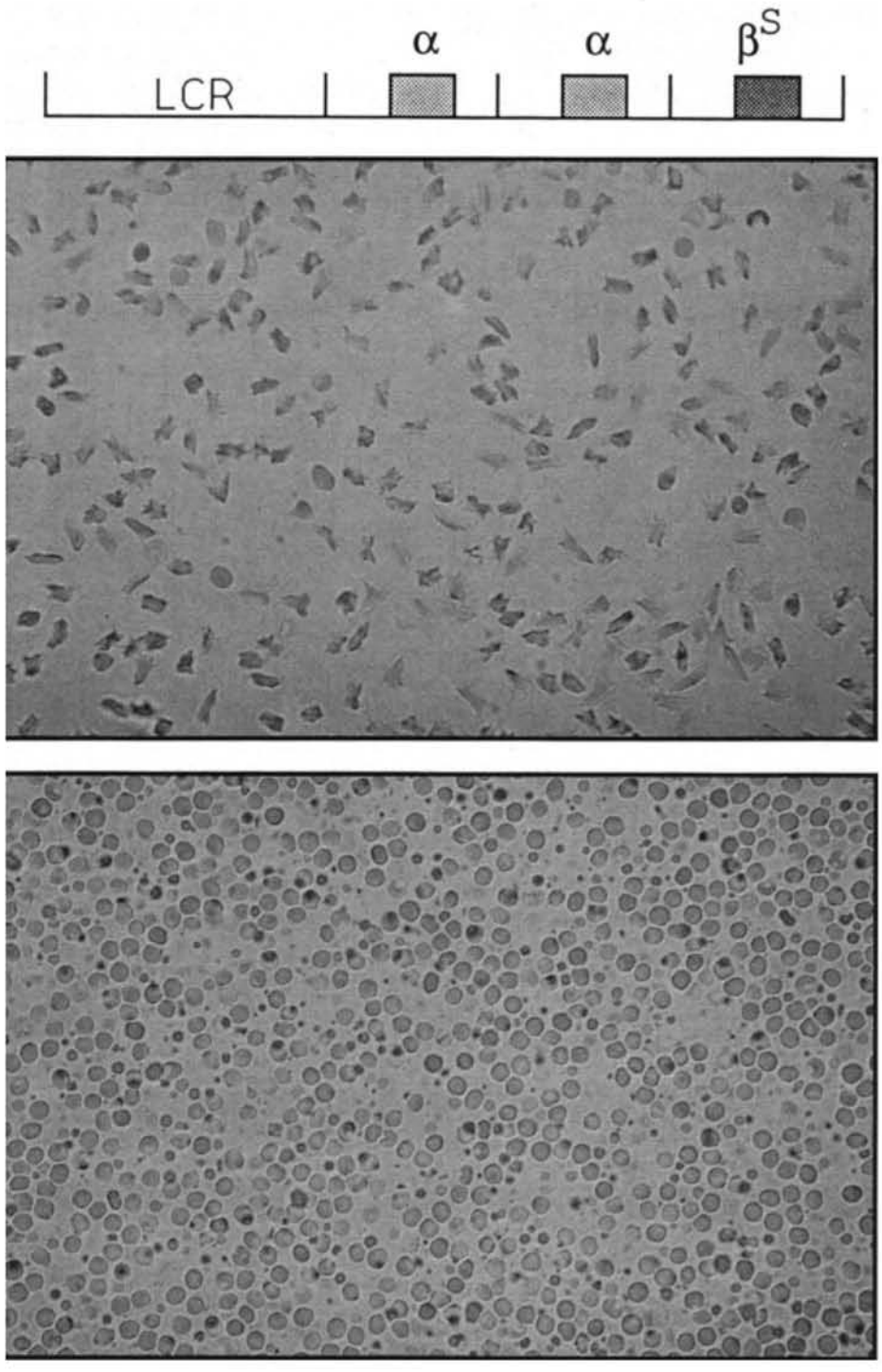

Fig. 5. Sickle cell disease in transgenic mice. The *top line* shows the arrangement of genes and the LCR that was injected into fertilized mouse eggs to obtain transgenic mice. The *top panel* shows sickled cells from one of the transgenic mice [26]; the *bottom panel* shows control nontransgenic red blood cells

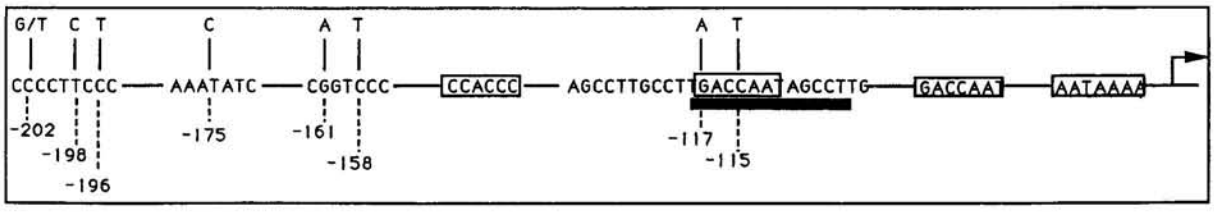

Fig. 6. Summary of mutations occurring in the γ-globin promoter resulting in HPFH phenotypes (see [44])

[21] which reduces affinity for the transcription factor CP1. The −117 mutation associated with Greek HPFH (40%) has been reported to cause reduced binding of the erythroid-specific factor NF-E3 [36]. These findings suggest a model for γ silencing in which factors binding to the distal CAAT box (at −115) compete for interaction with factors bound to upstream promoter sequences preventing the proximal CAAT box (at −87) from forming such interactions. The distal CAAT box is located outside the normal optimal position for CAAT elements, and this is likely to prevent it from functioning as an effective positive promoter element. One would expect this type of silencing mechanism to depend on the topology of the promoter region and it is also likely to be affected by the creation of extra factor binding sites in the upstream sequences. Such sites may partially bypass the competition between the proximal and distal CAAT boxes, resulting in suboptimal transcription. Indeed, a second group of mutations, upstream of −150, result in new or improved binding sites for transcription factors, e.g. Sp1 [16, 28] and GATA-1 [35, 37].

Activation of γ transcription in the nondeletion HPFHs is associated with down regulation of the β-gene. The reduction in β expression (to around 60% in Southern Italian HPFH) is approximately equivalent to the rise in expression of the *cis*-linked γ-globin gene, with only a slight reduction in overall transcriptional output from the locus [24, 62]. This suggests that competition is taking place between the genes and that this is tightly linked to the process of transcription.

However, a drastic reduction or loss of β transcription due to point mutations and deletions in the β-promoter does not significantly increase γ expression (less than 5%; Fig. 7) [44]. Clearly, a γ-gene exerts a negative effect on the β-gene (coupled to transcription) but this effect is not reciprocal. Some β-gene deletions show higher levels of γ expression (Fig. 7) but these deletions are always large (>10 kb). Of these, the Aγδβ-thalassaemias all have deletions which extend into the region of γ transcription. They are uninformative for competition models because enhancers found near the deletion breakpoints may be responsible for the high level, pancellular γ expression observed in the deletion HPFHs [1, 15]. Some increased γ expression is also observed in the δβ- and Dutch β-thalassaemias, but the broad range of values between patients with the same deletion and the heterocellular distribution of γ-protein among the red cells suggest that the increase in γ expression is not solely at the transcriptional level. This is supported by the observation that *non*transcriptional defects (e.g. RNA processing) in heterozygous β-thalassaemias cause elevated levels of γ-chains (up to 5%). Selection of a small proportion of cells expressing γ is a likely mechanism for this increase. Deletion of the δ-gene (which is normally expressed at only 2%−3% of the level of β) also does not seem to be required for the γ-expression observed in the δβ-thalassaemias, since it is intact in Dutch β-thalassaemia, which has a very similar phenotype. Instead, the requirement appears to be a minimum size of deletion (>10 kb). Probably these large deletions perturb the chromatin structure of the locus, resulting in a small increase in γ transcription which is further amplified by the chain imbalance. In conclusion, the genetic data show that strong down-regulation of the β-gene can result from an increase of γ-gene transcription, while there does not seem to be any significant link between transcription of the β-gene and silencing of the γ-genes in adult life.

Transgenic Mice. Attempts to study switching of globin genes have also made use of transgenic mice as a model system. Mice do not possess separate foetal globin genes but instead switch directly from embryonic to adult β-globin expression at 11−13 days of gestation (Fig. 2).

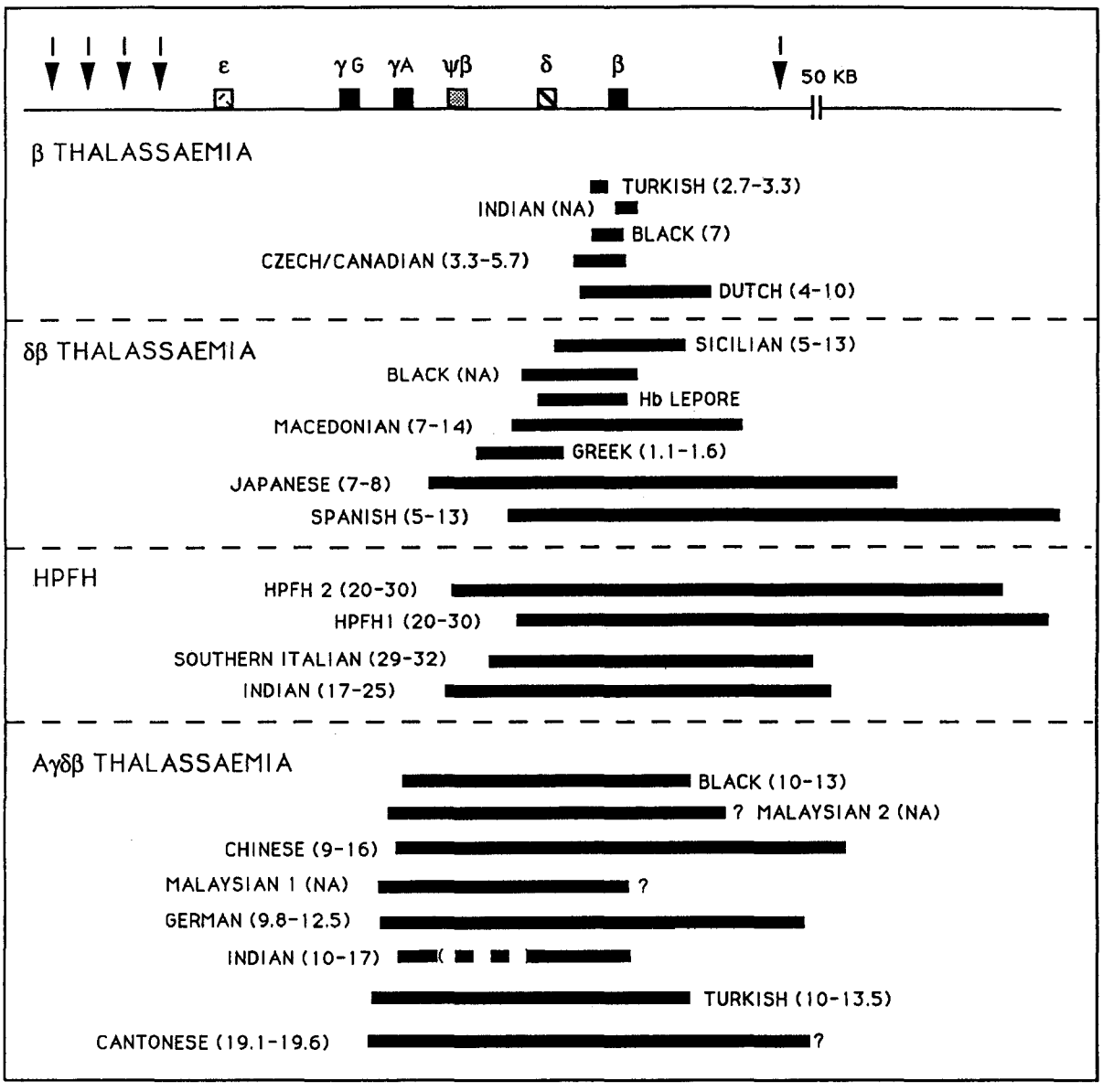

Fig. 7. Schematic representation of the different deletions occurring in the β-globin locus in thalassaemias and HPFHs. *Black bars* indicate the size of the deletion; *numbers in parentheses* indicate the levels of γ-globin expression in heterozygotes

The developmental regulation of the human ε-gene has been analysed in both embryonic stem cells and transgenic mice. In mice the ε-gene is expressed at high levels during the embryonic stage only when linked to the LCR and is completely silenced thereafter [33, 46, 51]. Based on the studies by Cao *et al.* deletion mutants lacking the −200 to −300 promoter region show a small increase in ε expression in adult transgenic mice but the low level indicates that other sequences may also be involved in silencing ε (P. Fraser, unpublished observations).

The human γ-transgene without the LCR is expressed like the mouse embryonic genes [7, 32]. It was initially reported that linkage to the LCR resulted in γ expression at all developmental stages and that the γ-gene was silenced in adult mice only when the β-gene was also present. This appeared to support a competition model where the β-gene is required for silencing of the γ-gene [2, 14]. However, a different result was obtained when the single γ-gene experiments were carried out on animals carrying only one or two copies of the LCR-γ-gene con-

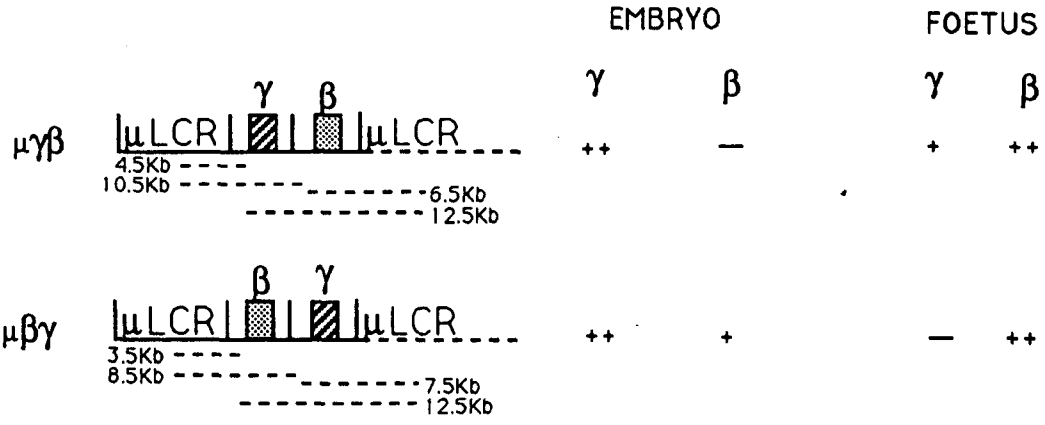

Fig. 8. Microlocus (μLCR) *γβ* and *βγ* constructs [29]. Genes are represented as *shaded boxes*. All genes are in the same trancriptional orientation, 5′ to 3′, with respect to each other and the LCR. The *dotted LCR lines* indicate the situation in multicopy animals to illustrate the distance from a promoter to a 5′ and 3′ LCR. These distances are indicated by *dotted lines* below the constructs. *Plus* and *minus symbols* indicate high, medium, and very low levels of expression

struct. *γ* expression persisted in the early foetal liver, but was silenced at adult stages, independent of the presence of the *β*-gene [12]. Transcription of the LCR-linked *γ*-gene can therefore also be blocked completely by stage-specific negative regulators acting on the sequences immediately flanking the gene, and this removes the basis of the argument that the *β*-gene would be needed for *γ* silencing. The elements responsible for *γ* silencing have not yet been identified but the mutations associated with the nondeletion HPFHs suggest that at least the sequences around the distal CAAT box are likely to be involved (see above). The availability of a transgenic mouse model for *γ*-gene silencing should allow this to be tested and possibly lead to novel approaches for treating thalassaemia and sickle cell anaemia. If *γ*-gene expression were understood at the level of the transcription factors, it might be possible to develop novel therapies that could specifically interfere with the adult suppression of the *γ*-gene and alleviate the clinical problems associated with severe chain imbalance or sickling.

Linkage of the adult *β*-gene to the LCR results in inappropriate expression at the embryonic stage [2, 3, 14, 29, 33], albeit at a low level. Placing a *γ*-gene or a human *α*-globin gene between the *β*-gene and the LCR blocks this expression [2, 14, 29], supporting the idea that competition plays a role in preventing premature *β* expression. However, when the order is reversed and the *β*-gene is placed in the first position, it is expressed at a level similar to that observed for the *β*-gene in the absence of the *γ*- or *α*-gene (Fig. 8) [29]. Silencing of the *β*-gene at the embryonic stage is therefore not caused by reciprocal competition only, but relative distance between the LCR and the genes (i.e. position and polarity) is also important.

Polarity in the locus has long been suggested by the fact that the genes are arranged in the order of their expression during development. The order of the genes is conserved among mammals but there is some divergence in the other vertebrate loci. In chicken, the embryonic *ε*- and *ϱ*-genes are located at opposite ends of the locus, with the adult *β*-genes between them. However, it is important to note that the chicken *β*-globin LCR may have been split as part of an *ε* translocation such that part of it is located between the *β*- and *ε*-genes [8, 47] and that the *ε*-gene contributes only 20% of the total embryonic haemoglobin compared to 80% for *ϱ* [5].

The data reviewed above indicate that developmental regulation of the human

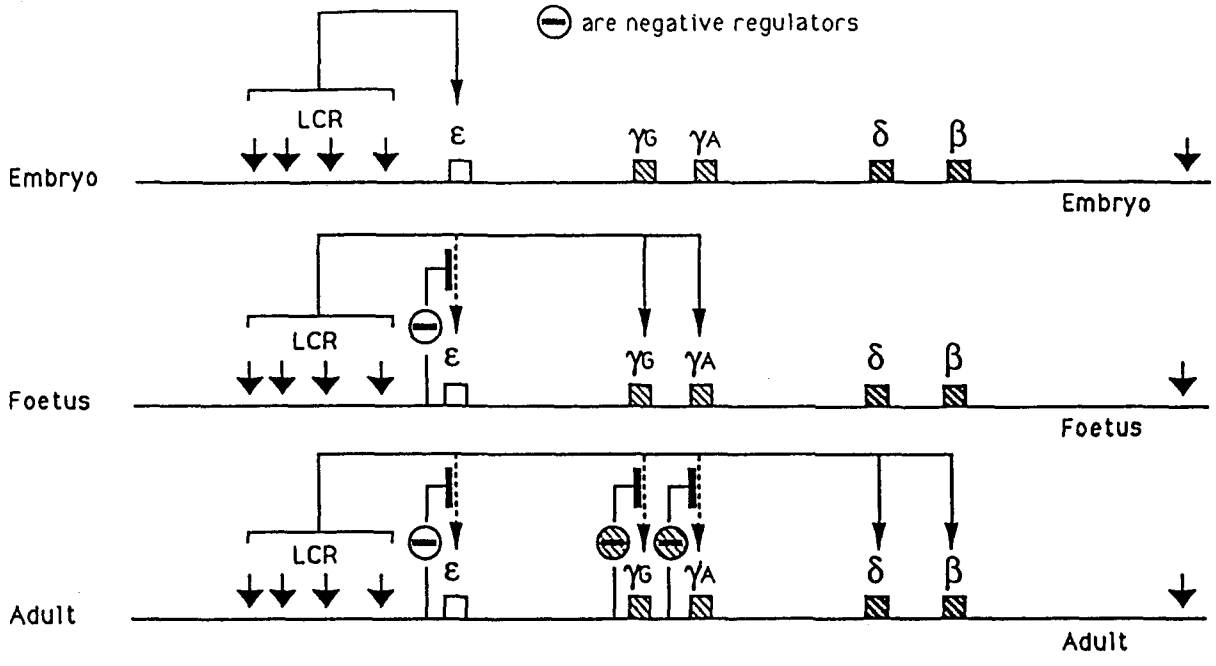

Fig. 9. Model for stage-specific regulation of the genes of the β-globin locus. *Solid lines* indicate activation of genes by the LCR. The symbol θ indicates stage-specific negative factors silencing the gene. The location of these is not accurate and there may be more than one factor for each gene

β-globin locus is a complex process which centres around developmentally specific suppressors and the polarity of the locus (Figs. 9, 10). The earliest gene to be activated, the ε-gene, is also the one closest to the LCR. The γ- and β-genes may be suppressed by competition with ε; alternatively, or in addition, the γ- and β-genes may bind embryonic stage-specific factors which keep their promoters suppressed. The ε-promoter is silenced in the foetal liver by one or more suppressor factors, negating its competitive ability (Fig. 9). As a result the γ-genes are expressed, and they in turn keep expression of the β-gene suppressed by competition. The γ-genes are switched off during the period around birth, again by stage-specific negative regulators, and as a consequence the β-gene is activated and expressed in the bone marrow. We propose that loop formation between regulatory elements is the crucial parameter to explain the suppression of the late genes by the early genes at early stages but not vice versa.

The frequency of interaction between the promoters and the LCR will depend on the effective volume in which these elements operate. This effect would be most pronounced if the LCR and the genes were all present on one structural chromatin loop several times the distance between the LCR and the genes. The fact that the LCR controls DNase hypersensitivity of the β-globin locus over at least 150-kb [19] suggests that the entire β-locus may be present on one very large chromatin loop. If we assume that to be the case, the frequency of interactions between any of the promoters with the LCR would be proportional to their effective concentration relative to the LCR (Fig. 10). On basis of ring closure probabilities with naked DNA, the effective concentration of two points on the DNA will be related to the volume of a sphere and will be proportional to the power of 3/2 of the distance. Applying the rule to the β-locus, the β-gene is twice as far as the Gγ-gene from the HS 2 enhancer of the LCR. Therefore, the β-gene occupies an approximately eight-fold larger volume relative to the HS-2 enhancer of the LCR than the Gγ-gene, which should give it a three-fold lower fre-

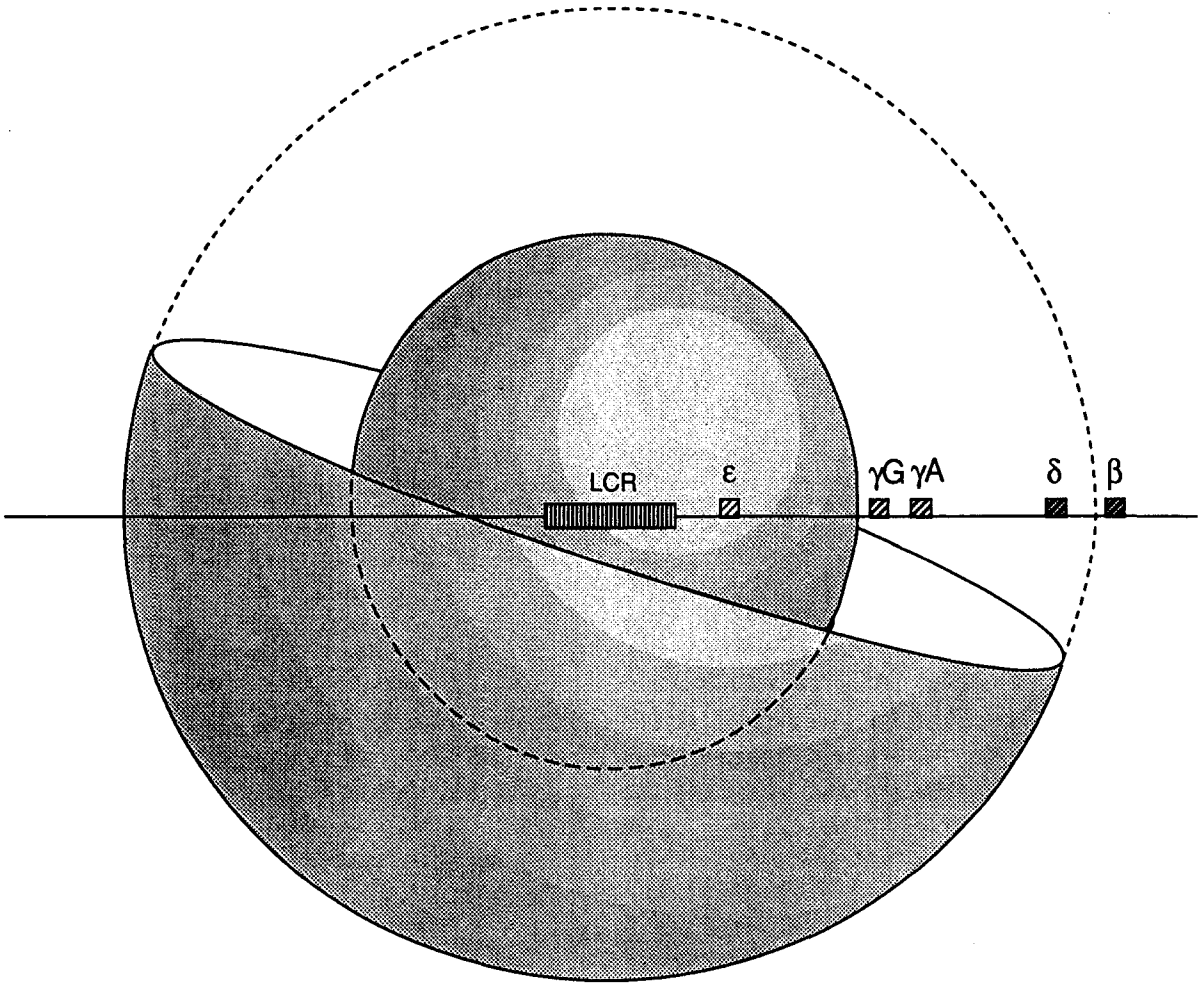

Fig. 10. Schematic representation of the relative volumes occupied by the Gγ- and β-genes relative to the LCR. For simplicity of presentation the LCR is shown as a fixed point in the centre of the sphere. Only half the β-globin gene outer sphere is shown

quency of interaction with the LCR (Fig. 10). This effect will work in favour of the proximal gene, decreasing the affinity differences required for competition, but it will work against the distal gene. Distal genes would be incapable of suppressing upstream genes under similar circumstances unless the downstream gene promoter increased its affinity by several orders of magnitude relative to the upstream gene. The transgenic mouse data on the expression of the β-globin gene at the embryonic and foetal/adult stages argue strongly against this possibility. Instead, the problem is solved by local suppression of the upstream promoters to allow expression from the downstream gene (Fig. 9). Experiments to substantiate or disprove this prediction are presently in progress.

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