fluidity in the interconnecting helix (*15*). Thus, Ca^{2+} serves to organize and stabilize domain structure in a conformation that can bind the target, while the central helix remains flexible, an essential condition for target recognition. Target binding further stabilizes domain structure, which should and does increase Ca^{2+} affinity (*16*). We believe that the Ca^{2+} signal restricts the available conformational states of CaM to those that are most favorable to target recognition and activation. The modulation of the inherent plasticity of CaM by the different ligands is a key element of molecular recognition and the mechanism of signal transduction.

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13. The amphipathic helical CaMKII and smMLCK peptide segments portray two variations, with the overlapped hydrophobic residues Leu^{299} and Trp^{800} near the NH_2-terminal ends (Figs. 1 and 3, A and B). A third viable target sequence would be a hybrid of the two segments with an eight-residue sequence separating the two hydrophobic residues—one in an identical position as Leu^{813} of the smMLCK peptide near the COOH-terminal end and the other in an equivalent position to that of Ile^{303} of the CaMKII peptide, which is nestled in the COOH-domain. The presence of hydrophobic residues in all four key positions would represent the fourth and ideal target sequence. The wide hydrophobic cavity in both domains of Ca^{2+}-CaM (Figs. 2B and 3B), combined with adjustment of the domain geometry described in the text, makes it possible for Ca^{2+}-CaM to accommodate these variations.
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15. The ease with which the central helix or expansion joint is deformable is indicated in the extent of disorder or the high thermal parameter of this region in the refined crystal structures. The expansion joint region in the complex with the CaMKII peptide is totally disordered, as is evident from the absence of electron density. Although the electron density of the expansion joint in the complex with the smMLCK peptide in the four independent molecules contained in the asymmetric unit was observed for the most part, this segment exhibited the highest averaged isotropic thermal *B* factor (*9*). In the native unbound 1.7 Å structure, this region of the central helix (from residues 73 to 83) has an averaged *B* factor 1.6 times greater than that of the entire molecule (*7*). The high thermal motion of the central helix was first noted in the 2.2 Å structure of native Ca^{2+}-CaM (*6*).
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17. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Restoration of HIV-Specific Cell-Mediated Immune Responses by Interleukin-12 in Vitro

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Peripheral blood mononuclear cells (PBMCs) from many asymptomatic individuals infected with human immunodeficiency virus–type 1 (HIV) are unresponsive as measured by in vitro T cell proliferation and interleukin-2 (IL-2) production to influenza virus and synthetic peptides of HIV envelope (Env). Strong influenza virus– and Env-stimulated IL-2 responses and T cell proliferation were restored when cultures were stimulated in the presence of IL-12. Interferon-γ production by PBMCs from HIV seropositive (HIV^+) patients was also restored with IL-12. Furthermore, in vitro antigen-specific production of IL-2 and proliferation of PBMCs from HIV^- donors were suppressed by antibody to IL-12, but were not enhanced by addition of exogenous IL-12. Thus, IL-12 may be limiting in PBMCs from HIV^+ but not HIV^- individuals. These findings demonstrate that IL-12 can restore HIV-specific cell-mediated immunity in vitro in HIV-infected individuals and suggest a potential use of IL-12 in augmenting the diminished immunologic functions associated with HIV infection.

The recently discovered cytokine IL-12 has been reported to increase (i) natural killer cell and cytotoxic T lymphocyte (CTL) activity (*1*); (ii) T cell proliferation (*2*, *3*); and (iii) the production of interferon-γ (IFN-γ) (*4*). We previously demonstrated that the progression of HIV disease in HIV-infected (HIV^+) individuals is associated with a switch from a T helper 1 (T_H1)–like cytokine profile (high IL-2, low IL-4, and low IL-10 production) to a T_H2-like cytokine pattern (low IL-2, high IL-4, and high IL-10 production) (*5*, *6*). This change in cytokine profile is predictive of a decline in the number of peripheral blood $CD4^+$ T cells (*7*), as well as of the time until diagnosis of acquired immunodeficiency syndrome (AIDS) and time to death (*7*). We recently demonstrated that IL-4 and IL-10 antibodies can reverse the T_H1-to-T_H2 switch in vitro and restore in vitro the ability of the PBMCs of HIV^+ individuals to produce IL-2 (*6*). IL-12 can bypass the inhibitory effect of IL-10 on the induction of a T_H1-like function (*4*). Therefore, on the basis of the above findings we asked whether the defective T_H1-like cell-mediated responses of PBMCs from HIV^+ individuals could also be reconstituted in vitro by IL-12.

The PBMCs of 40 HIV^+ individuals that were unable to produce IL-2 in response to five synthetic peptides corresponding to antigenic regions of the envelope (Env) of HIV-1 (*8*) in vitro were stimulated with Env in the presence of IL-12 (*9*). The data obtained from three representative individuals are shown in Fig. 1A (panels a through c), along with the response generated by PBMCs from an uninfected, healthy control donor (panel d). Cultures from all three of the HIV^+ individuals responded strongly to Env only when IL-12 was added. In contrast, IL-12 did not elevate above the background the IL-2 response to Env of the HIV^- culture or induce IL-2 production by HIV^+ cultures in

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response to the nonantigenic peptide p23 (*10*). An increase in IL-2 responses to Env, defined as a greater than threefold increase above the basal level, was observed in 28 of 40 (70%) HIV$^+$ individuals tested. No Env-stimulated IL-2 production was seen in any of the nine HIV$^-$ individuals tested with or without IL-12. A similar IL-12–induced increase in Env-stimulated T cell proliferation by PBMCs of HIV$^+$ patients was observed (Fig. 1B).

To determine whether IL-12 would also increase T helper cell function to non-HIV antigens in these HIV$^+$ individuals, we stimulated the PBMCs of 47 HIV$^+$ individuals with the influenza virus (FLU), human lymphocyte antigen (HLA)–disparate PBMCs (ALLO), or phytohemagglutinin (PHA). The data of Fig. 2, A through C, illustrate that the proliferative responses to FLU of three representative HIV$^+$ individuals was increased approximately 10-fold above the background, and their ALLO response (which was potent without IL-12) was increased two- to fivefold. IL-12 induced a greater than threefold increase in FLU-stimulated proliferation in 32 of 47 (68%) HIV$^+$ individuals tested. The intact FLU responses of HIV$^-$ controls were not further increased by IL-12, which illustrates that IL-12 augmented the deficient responses of the HIV$^+$ individuals. Proliferation of unstimulated cultures was not increased by IL-12. Similar results were obtained for FLU-, ALLO-, and PHA-stimulated IL-2 production, as the FLU response of the HIV$^+$ patient shown was increased 10-fold and the ALLO and PHA responses were increased fourfold by IL-12 (Fig. 2, G and H). Responses to the same stimuli were not increased in the HIV$^-$ control (Fig. 2, I and J). No correlations were observed between the IL-12–induced elevation of IL-2 production and proliferation and the clinical stage, the number of CD4$^+$ T lymphocytes, or the CD4:CD8 ratios of the patients. IL-12–induced elevation of IL-2 production and proliferation was most frequently observed, however, in patients with a type 2 cytokine pattern (29 out of 32 or 90%). We conclude that IL-12 is limiting in cultures of PBMCs from HIV$^+$ but not from HIV$^-$ individuals.

Interleukin-12 has also been shown to stimulate the production of IFN-γ from T cells and natural killer (NK) cells (*4*). We measured PHA-stimulated IFN-γ production by PBMCs from 10 HIV$^+$ individuals in the absence or in the presence of IL-12 (20 U/ml) (*11*). As shown in Fig. 3A, IFN-γ production was diminished (defined as <1000 ng/ml) in 8 out of 10 HIV$^+$ patients but was augmented 5- to 20-fold after incubation with IL-12 in six of these eight patients (Fig. 3B). PHA-stimulated IFN-γ production was not defective (>1000 ng/ml) in any of five HIV$^-$ controls tested (Fig. 3C) and was not appreciably augmented by IL-12 in any of the cultures (Fig. 3D). The amount of IL-10 detected by enzyme-linked immunosorbent assay (ELISA) in the supernatants of PHA-stimulated PBMCs from these patients was reduced to one-half to one-third of previous levels by the addition of IL-12 to the same cultures.

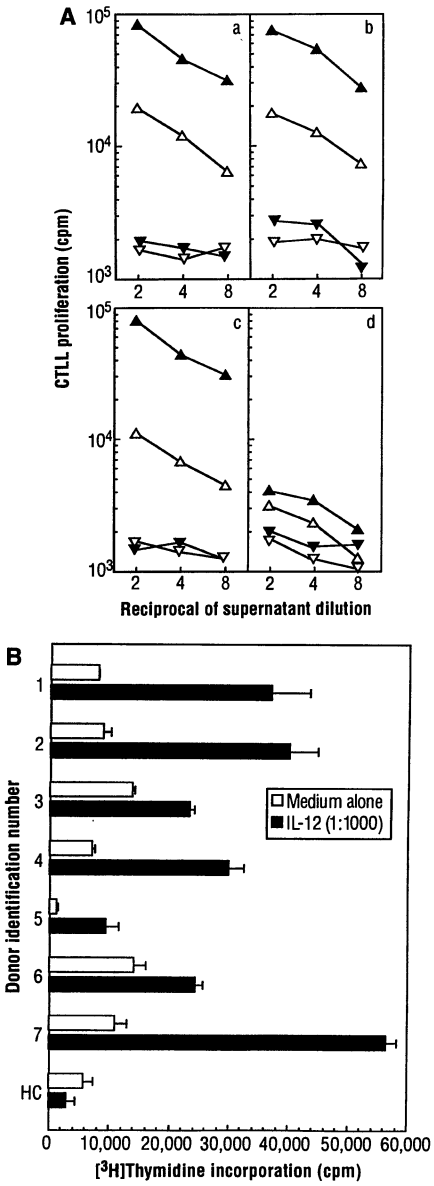

Fig. 1. (**A**) IL-2 production in response to stimulation of PBMCs from three HIV$^+$ individuals (panels a through c) and from one HIV$^-$ individual (panel d) in response to a pool of five Env peptides cultured with (▲) or without (△) IL-12. IL-2 production in unstimulated cultures with (▼) or without (▽) IL-12 is also shown. The five Env peptides used were T1, T2, Th4.1, P18 IIIB, and P18MN (*8*). (**B**) Proliferation of PBMCs from seven HIV$^+$ individuals (numbered) and from one HIV$^-$ individual (HC) in response to the same pool of Env peptides cultured with (solid bars) or without IL-12. Standard errors bars are indicated. The background (<500 cpm) was subtracted.

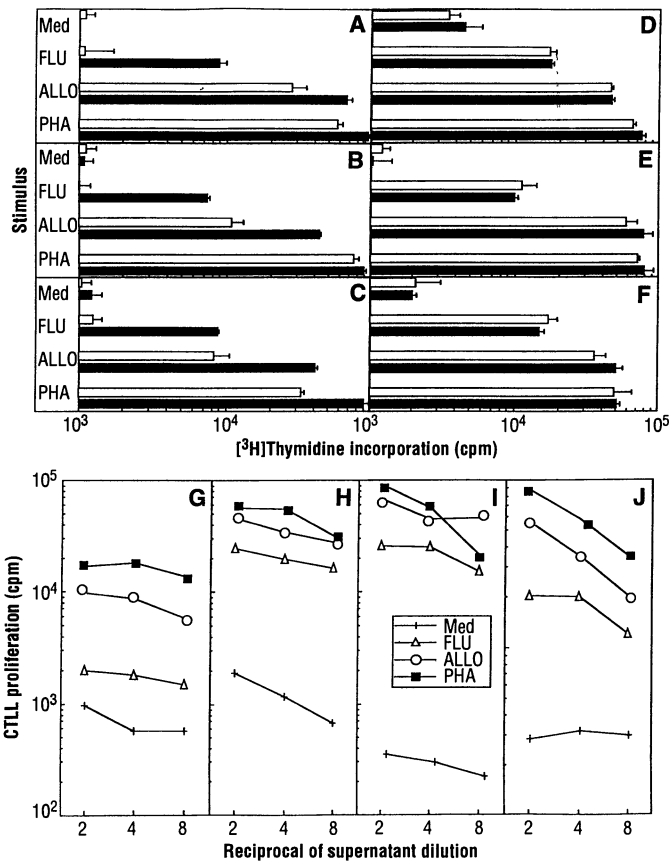

Fig. 2. Proliferation of PBMCs from three HIV$^+$ individuals (**A** through **C**) and from three HIV$^-$ controls (**D** through **F**) in response to FLU, ALLO, and PHA, cultured with (solid bars) or without IL-12. Med indicates unstimulated cultures; FLU indicates cultures stimulated with influenza A virus; ALLO indicates cultures stimulated with irradiated (50 Gy) HLA-disparate PBMCs; PHA indicates cultures stimulated with phytohemagglutinin. Standard error bars are indicated. (**G** through **J**) Antigen- and mitogen-stimulated IL-2 production by PBMCs from an HIV$^+$ individual (G and H) and an HIV$^-$ control (I and J) stimulated in the absence (G and I) or presence (H and J) of IL-12 (1:1000 dilution).

Earlier studies demonstrated the ability of IL-12 to elevate the cytolytic activity of murine (*12*) and human (*13*) NK cells; IL-12 has also been found to enhance murine allogeneic CTL activity that is H-2–specific (*14*). Therefore, we tested whether IL-12 would increase the NK activity of PBMCs from the HIV$^+$ individuals. We observed that IL-12–supplemented cultures of PBMCs from eight HIV$^+$ and four HIV$^-$ individuals increased lysis of K-562 cells by approximately 10-fold, thus supporting the published report of IL-12–enhanced NK cell activity (*13*). Preliminary data also suggest that HIV-specific and FLU-specific CTL activity of PBMCs from HIV$^+$ individuals is enhanced in vitro by IL-12 (*15*).

Conversely, to determine whether IL-12 antibody could inhibit T cell proliferation, PBMCs from HIV$^-$ donors were stimulated in the presence of a neutralizing polyclonal antibody directed toward human p40 of IL-12 during antigenic stimulation (*16*). Figure 4 summarizes the data from one of five experiments with similar results. The proliferative responses to FLU and ALLO were greatly reduced by antibodies to IL-12. Thus, although IL-12 is not limiting in PBMCs of uninfected individuals, it is necessary for T cell proliferation.

The activity of IL-12 in the development of T_H1 T cells has been suggested by recent in vitro and in vivo studies. Hsieh *et al.* (*17*) demonstrated that IL-12 can induce the in vitro differentiation of naive T cells (T_H0 cells) into T_H1 cells. Using murine models of leishmaniasis, Sypek *et al.* (*18*) and Heinzel *et al.* (*19*) reported that IL-12 induces a T_H1 response in vivo. An association between IL-12 and T_H1-like functions in humans was suggested by our previous findings and the recent observation that production of *Staphylococcus aureus*–stimulated IL-12–p40 by PBMCs of HIV$^+$ individuals is reduced to one-tenth that of healthy controls (*20*). Our previous findings (*6*) are consistent with the hypothesis that a major portion of the immune deficiency seen in HIV$^+$ individuals is due to an imbalance in type 1 versus type 2 cytokine production, in which type 2 cytokines become more prevalent in the progression to AIDS (*6*, *21*). Those results are also consistent with the recent observation that helminthic parasitic infections, which cause a similar shift from a type 1 to a type 2 cytokine profile, lead to diminished CD8$^+$ CTL activity against viral antigens and a delay in clearance of virus in vivo (*22*).

Our results indicate that IL-12 can augment production of cytokines associated with the T_H1 phenotype in cultures of PBMCs from HIV$^+$ patients. Antigen-specific induction of IL-2 has not been previously reported for IL-12 and may represent an important aspect of in vivo IL-12 function. Whether this activity and its associated responses are due to the activation of T_H1 T cells or reflect the known activities of IL-12 on existing CD4$^+$, CD8$^+$, or NK cells remains to be determined.

Our data show that IL-12 induces IL-2 and IFN-γ production and proliferation of PBMCs from HIV$^+$ patients after antigenic stimulation. Our data also demonstrate that antigenic stimulation in the presence of IL-12 antibodies can induce T helper cell functional defects in PBMCs from HIV$^-$ individuals similar to those seen in HIV$^+$ individuals. Thus, our findings imply that in HIV infection a type 1 response can be restored in vitro by IL-12. It is possible that these responses are due to the differentiation of naive cells into T_H1 cells, as has recently been suggested by Manetti *et al.* (*23*). However, it is unlikely that IL-12 could induce a FLU-specific type 1 response from naive cells without priming with FLU antigens. A recent report suggested that once a type 2 response is established during *Leishmania* infection of susceptible mice, administration of IL-12 does not reverse but may delay progression of the disease (*19*). These findings question whether IL-12 can directly induce a shift from a type 2 to a type 1 response.

An alternate possibility to a type 2 to type 1 switch is that exogenously added IL-12 may induce proliferation, IL-2 production, and IFN-γ production by acting directly on existing populations of cells under conditions in which endogenous IL-12 production is limiting. Gateley *et al.* have shown that IL-12 in the presence of small amounts of IL-2 can promote proliferation of resting PBMC cultures (*3*). The combination of IL-4 and IL-12 has also been shown to promote proliferation of NK cells (*24*). The synergy of IL-12 with IL-2 in the induction of IFN-γ (*25*) may also account for the enhanced IFN-γ production measured in IL-12–treated cultures of PHA-stimulated PBMCs. Both T and NK cells have been shown to produce IFN-γ in response to the combination of IL-12 and IL-2 (*4*). IL-12 also elevates NK cell activity in cultures of murine spleen cells and human PBMCs and increases murine CTL responses in vitro. Our results verify those of Chehimi *et al.* (*13*), which demonstrate IL-12–induced enhancement of human NK activity.

Whether by direct effect on existing T or NK cells or through the induction of T_H1 T cell populations, the efficacy of IL-12 in augmenting the immune function of PBMCs from HIV$^+$ patients raises the possibility of using IL-12 as an immune modulator to augment the defective cell-mediated functions associated with HIV infection (*26*). Further studies of the mechanism of action, efficacy, and toxicity of IL-12 in in vivo models will be critical in evaluating its potential for restoring type 1 function.

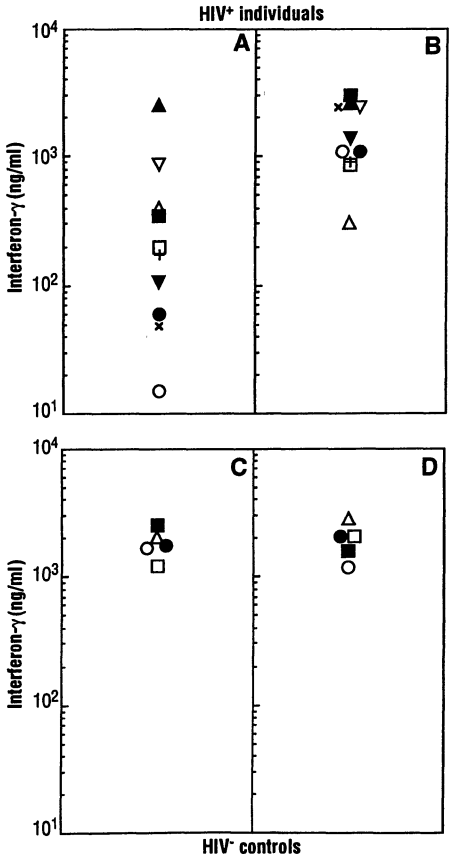

Fig. 3. PHA-stimulated production of IFN-γ by PBMCs from 10 HIV$^+$ individuals (**A** and **B**) and from 5 HIV$^-$ individuals (**C** and **D**). PBMCs were cultured without (A and C) or with (B and D) IL-12. Different symbols indicate IFN-γ responses by PBMCs from different individuals.

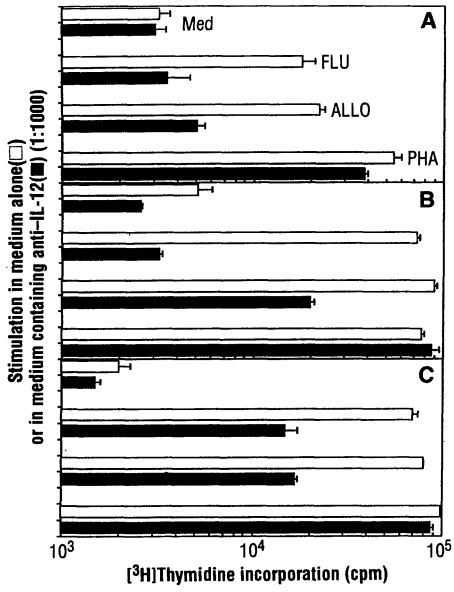

Fig. 4. Effect of incubation of PBMCs from HIV$^-$ controls with a neutralizing p40 IL-12 antibody (anti–IL-12) during antigen or mitogen stimulation. (**A**), (**B**), and (**C**) represent PBMCs from three different donors, respectively.

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9. The PBMCs of HIV$^+$ individuals or of HIV$^-$ controls resuspended at 3 × 10^6 per milliliter in RPMI 1640 (Gibco, Grand Island, NY) were either unstimulated or stimulated in vitro for 7 days with a mixture of five previously described antigenic synthetic peptides from Env of HIV-1 (T1, T2, Th4.1, P18IIIB, and P18MN) (*8*) or the Env nonantigenic peptide p23 at a final concentration of 5 μM/ml in sterile, 96-well flat-bottom culture plates (Costar, Cambridge, MA). In a second series of experiments, PBMCs were either unstimulated or stimulated for 7 days with influenza A virus (A/Bangkok/RX73 H3N2) (final concentration, 1:500), with a pool of irradiated [50 Gy (units of absorbed dose of ionizing radiation)] PBMCs from multiple donors (resuspended at 2 × 10^6 per milliliter) (ALLO), or with PHA (Gibco) (final concentration, 1:100). The PBMCs were stimulated in the absence or the presence of 20 U of recombinant human IL-12 per milliliter. For the IL-2 production assays, the IL-2 receptor monoclonal antibody TAC was added to the cultures (2 μg/ml) to prevent consumption of the IL-2 produced. Supernatants were harvested after 7 days and serially diluted, and the amount of IL-2 produced was assessed in a bioassay with the IL-2–dependent cell line CTLL as described (*5*). In the proliferation assays, the cell cultures were cultured for 5 days in 96-well flat-bottom plates, pulsed with 1 mCi of [^{3}H]thymidine, and harvested after 18 hours.
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11. PBMCs (3 × 10^6 per well) in a 24-well LINBRO plate were stimulated for 48 hours with PHA diluted 1:100 (Gibco). The PBMCs were stimulated in medium alone or in medium containing 20 U of recombinant IL-12 per milliliter. The amount of IFN-γ present in the supernatants was determined with a two-step ELISA assay. Briefly, Immulon plates (Dynatech Labs, Chantilly, VA) were coated overnight at 4°C with 100 μl per well of the A35 IFN-γ antibody (DNAX Research Institute) (final concentration, 5 μg/ml). Plates were washed five times in phosphate-buffered saline (PBS)–Tween and blocked with 150 ml of 10% fetal calf serum in PBS. Supernatants were diluted through three serial dilutions (1:2 to 1:8) in PBS-Tween, which contained the IFN-γ nitroiodophenyl-conjugated antibody B27 (final concentration, 5 μg/ml). A 100-μl aliquot of each sample was added to each well, and a standard for IFN-γ was run concurrently. Plates were washed with PBS-Tween five times. Horseradish peroxidase–conjugated antibody J4-HRP was diluted 1:3000 in PBS, and 100 μl was added to each well and incubated for 1 hour at 37°C. Plates were again washed with PBS-Tween. ABTS peroxidase substrate was diluted 1:1 with H_2O_2 (Kirkegaard-Perry, Gaithersburg, MD), and 100 μl of this mixture was added to each well. Plates were developed for 15 min and then read in an ELISA reader at a wavelength of 405 Å. Values for IFN-γ were calculated from a standard curve.
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Dense Nonsymmetrical DNA Methylation Resulting from Repeat-Induced Point Mutation in *Neurospora*

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Cytosine methylation has been implicated in epigenetic control of gene expression in animals, plants, and fungi. It has been assumed that all methylation in eukaryotes is at symmetrical sequences such as CpG/GpC, because this can explain perpetuation of methylation states. Here the bisulfite genomic sequencing method was used to examine methylation in DNA from a *Neurospora* gene exposed to repeat-induced point mutation. 5-Methylcytosine was not limited to symmetrical sites and individual molecules showed different patterns and amounts of modification. The methylation extended beyond the mutated region and even beyond the edge of the duplicated segment.

A fraction of the cytosines in the DNA of many organisms becomes methylated after replication. This methylation is not randomly distributed. Some sequences, such as the CpG islands of animals, are rarely if ever methylated (*1*). Others are methylated under certain circumstances, such as when the sequence is inherited from the mother rather than the father (*2*), and still others appear methylated in all tissues and at all times (*3*).

Neither the precise function nor the control of DNA methylation is understood in any eukaryote. In animals, most 5-methylcytosine (5mC) is found in the symmetrical sequence 5′CpG/3′GpC, and this is thought to be mediated by a maintenance methylase that preferentially recognizes and methylates hemimethylated sites. Such an enzyme could be responsible for propagating patterns of methylation (*4*). Indications of heterogeneous methylation in populations of cells thought to be clonally related suggest, however, that maintenance methylases cannot be solely responsible for persistence of methylation (*5–7*). There is evidence of substantial non-CpG methylation in plants (*8*) and fungi (*5, 9*) and of occasional non-CpG methylation in animals (*10, 11*). Nevertheless, it is generally assumed that all non-CpG methylation is in other symmetrical sequences, such as CpNpG (*12*), which might also be recognized by a maintenance methylase.

Results of Southern (DNA) hybridizations on DNA from the fungi *Neurospora crassa* and *Ascobolus immersus* suggested that methylation is not limited to symmetrical sites (*5, 9*). We have investigated this possibility directly in *N. crassa*. Whereas most of the *Neurospora* genome appears devoid of DNA methylation, heavily methylated chromosomal regions have been identified. At least some such regions are relics of repeat-induced point mutation (RIP), a process operating in the sexual phase of the life cycle that detects sequence duplications and riddles them with G:C to A:T mutations (*13*). We investigated the methylation of a product of RIP to explore the possible connection between DNA methylation and RIP and to further our understanding of methylation in eukaryotes.

Information about the distribution of methylated cytosines has come almost exclusively from Southern hybridizations on genomic DNA with restriction enzymes that are sensitive to 5mC. The development of several genomic sequencing methods made it possible to assess the methylation status of any cytosine in genomic

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