complex of *Bacillus* fragment and DNA with a three-nucleotide 5' template overhang in which the DNA bound in symmetric orientations with both blunt and recessed 3' ends at the polymerase active site (C.M. and L.S.B., unpublished observation). Based on this observation, in complex I the 3' end of the template strand was phosphorylated to fix the orientation of the DNA. In complex II, the pseudo-symmetric sequence presents an identical template overhang in both orientations. DNA was incubated with a 10 mg ml⁻¹ protein solution in a 3:1 molar ratio. Crystals of the complex of *Bacillus* fragment and DNA were formed by hanging-drop vapour diffusion⁵ and transferred into a cryoprotectant solution containing 24% (w/v) sucrose before data collection.

Data collection and structure determination. Diffraction data were collected at 98 K using a RAXIS-IIC detector (Molecular Structure) on a Rigaku rotating anode X-ray generator. The crystals of the complex belong to the orthorhombic space group of $P2_12_12_1$ with unit cell dimensions $a = 86.2 \text{ \AA}$, $b = 93.2 \text{ \AA}$ and $c = 106.4 \text{ \AA}$, and contain one molecule per asymmetric unit. Data were processed using DENZO and SCALEPACK²³ (Table 2). Rigid-body refinement²⁴ of the apoenzyme coordinates⁵ provided the initial phases for the 9-bp *Bacillus* fragment–DNA complex data. Inspection of ($F_o - F_c$) and ($2F_o - F_c$) difference electron-density maps showed striking helical density for 9 bp of duplex DNA. The nucleotides were fit into this density using O²⁵ and the structure was refined with X-PLOR²⁴ using the new DNA force field and topology parameters²⁶ (Table 2).

The thumb subdomain (residues 507–585) is in a significantly different conformation from the apoenzyme structure and was rebuilt into difference electron-density maps updated during refinement. Residues 548–553, which were disordered in the apoenzyme structure, are seen in the *Bacillus* fragment–DNA complex and interact with the phosphate backbone (Fig. 1). The entire *Bacillus* fragment protein (residues 297–876) is fit in the electron density, except for 12 residues at the N terminus which are disordered. The average protein B-factor is 21 Å², and 26 Å² for the DNA. Density for the first two nucleotides of the template overhang at the active site is apparent, although the remaining 5' single-stranded nucleotides are disordered.

Catalysis in the crystal. Complex I or II co-crystals were transferred into a stabilizing solution (2.5% MPD, 100 mM MES buffer, pH 5.8, 60% (v/v) saturated ammonium sulphate) containing 33 mM ddTTP and 50 mM MnSO₄ (complex I) or 22.5 mM dATP and 50 mM Mg SO₄ (complex II) and incubated for 2–3 days at room temperature. The refined 9-bp *Bacillus* fragment–DNA coordinates were used as the initial model to phase the data from nucleotide triphosphate-soaked crystals. New electron density at the end of the DNA distal from the active site was observed in ($F_o - F_c$) and [F_o (nucleotide soaked) – F_o (unsoaked)] difference electron-density maps phased by the coordinates of the 9-bp complex (calculated using CCP4 (ref. 27)). This new density (Fig. 2) was consistent with the formation of one (ddTTP experiment) or two (dATP experiment) additional DNA base pairs. Positive and negative peaks in the ($F_o - F_c$) difference maps were also observed on the edges of the DNA base rings, indicating that the register of the initial modelled DNA sequence relative to the protein had changed. The DNA was rebuilt into the density, translocated by one base pair for the ddTTP experiment and two base pairs for the dATP experiment to fill the newly observed density at the distal end of the DNA. Additional base pair(s) representing the incorporated nucleotide(s) were modelled at the active site. Refinement of the model with the rebuilt, translocated DNA sequence caused an immediate 4% drop in the R-factor to 26% and eliminated the ($F_o - F_c$) peaks on the edges of the DNA base rings.

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erratum

Ataxin-1 with an expanded glutamine tract alters nuclear matrix-associated structures

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Figure 4c, d was reproduced with extraneous colour obscuring the gel patterns. The correct version is shown here. □

Ataxin-1 with an expanded glutamine tract alters nuclear matrix-associated structures

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Spinocerebellar ataxia type 1 (SCA1) is one of several neurodegenerative disorders caused by an expansion of a polyglutamine tract^{1,2}. It is characterized by ataxia, progressive motor deterioration, and loss of cerebellar Purkinje cells¹. To understand the pathogenesis of SCA1, we examined the subcellular localization of wild-type human ataxin-1 (the protein encoded by the *SCA1* gene) and mutant ataxin-1 in the Purkinje cells of transgenic mice³. We found that ataxin-1 localizes to the nuclei of cerebellar Purkinje cells. Normal ataxin-1 localizes to several nuclear structures ~0.5 µm across, whereas the expanded ataxin-1 localizes to a single ~2-µm structure, before the onset of ataxia. Mutant ataxin-1 localizes to a single nuclear structure in affected neurons of SCA1 patients. Similarly, COS-1 cells transfected with wild-type or mutant ataxin-1 show a similar pattern of nuclear localization; with expanded ataxin-1 occurring in larger structures that are fewer in number than those of normal ataxin-1. Colocalization studies show that mutant ataxin-1 causes a specific redistribution of the nuclear matrix-associated domain containing promyelocytic leukaemia protein⁴⁻⁷. Nuclear matrix preparations demonstrate that ataxin-1 associates with the nuclear matrix in Purkinje and COS cells. We therefore propose that a critical aspect of SCA1 pathogenesis involves the disruption of a nuclear matrix-associated domain.

To investigate the pathogenesis of the polyglutamine disorder SCA1, we examined the subcellular localization of wild-type human ataxin-1 and mutant ataxin-1 in the Purkinje cells of transgenic mice³. Because the ataxin-1 antibodies we used recognize both murine and human ataxin-1 (ref. 8), we used *SCA1* transgenic mice³ crossed onto a murine ataxin-1 null background⁹. Ataxin-1 localized to the nuclei, but not the nucleoli, of Purkinje cells in mice carrying either a wild-type (30 repeat) or mutant (82 repeat) *SCA1* allele at all ages examined (Fig. 1a, b). In Purkinje cells of transgenic mice expressing a wild-type *SCA1* allele, ataxin-1 localized throughout the nucleus and to multiple nuclear structures that were ~0.5 µm in size (Fig. 1c). Mutant ataxin-1 also localized throughout the nucleus but, in contrast to wild-type ataxin-1, it localized to a single large ~2-µm nuclear structure (Fig. 1d). The fraction of Purkinje cells containing the large nuclear inclusion of mutant ataxin-1 increased with age from 25% at six weeks of age to 90% at 12 weeks. Additionally, cells expressing mutant ataxin-1 often demonstrated invaginations in the nuclear membrane. The age of onset of ataxia, determined by cage behaviour for these mice, is 18 weeks (P.J.S. *et al.*, unpublished data); thus the changes in nuclear structure develop before the onset of ataxia.

We used immunohistochemistry to examine the subcellular

distribution of ataxin-1 in brain tissue from several human SCA1 patients. We found ubiquitin-positive nuclear inclusions of ataxin-1 in the nucleus pontis centralis, a region affected by the disease¹⁰. As an example, the results in Fig. 1e, f are from one patient with 29 and 82 CAG repeats. Examination of two other brain regions affected by SCA1, the olives and Purkinje cells, revealed substantial cell loss, making the identification of ataxin-1-positive neurons almost impossible. However, in the rare instances when a neuron could be identified in the inferior olive, the presence of ataxin-1 nuclear inclusions was demonstrated. Nuclear inclusions of ataxin-1 were not detected in brain regions unaffected by the disease. The results from SCA1 transgenic mice and patients demonstrate that ataxin-1 localizes to distinct nuclear domains in neurons. Furthermore, they demonstrate that an expanded form of ataxin-1 has a nuclear distribution that differs from that of wild-type ataxin-1.

In view of the nuclear localization of ataxin-1 and the nuclear changes induced by mutant ataxin-1 in Purkinje cells of transgenic mice and affected neurons of SCA1 patients, we examined the nuclear localization of ataxin-1 in a transfected cell line. We used monkey kidney COS-1 cells, because they do not express endogenous ataxin-1 yet readily express ataxin-1 upon transfection with *SCA1* constructs (Fig. 2a). When either a wild-type or expanded form of ataxin-1 was expressed in transfected COS cells, there was no evidence that ataxin-1 induced cell death. In these cells, trans-

Figure 1 Immunofluorescence of ataxin-1 within the nuclei of transgenic mouse Purkinje cells and SCA1 patient neurons. **a, c**, Localization of wild-type ataxin-1 with 30 repeats; **b, d**, localization of mutant ataxin-1 with 82 repeats in the Purkinje cells of transgenic mice. In **a** and **b**, a 10-µm series of confocal images are projected (scale bar, 15 µm). In **c** and **d** a single confocal Z scan is shown (scale bar, 5 µm). **e, f**, Ataxin-1 (**e**) and ubiquitin (**f**) immunostaining of neurons is shown in the nucleus pontis centralis from a SCA1 patient with an expanded allele of 82 repeats.

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fecting wild-type as well as mutant ataxin-1 localized to the nucleus and were excluded from the nucleoli (Fig. 2b–g). Most (75%) of the cells transfected with normal ataxin-1 showed a diffuse staining accompanied by many small structures of less than 1 μm in diameter (Fig. 2d). In contrast, 60% of COS-1 cells transfected with mutant ataxin-1 had ataxin-1-containing nuclear structures that were fewer in number and larger in size, sometimes exceeding 5 μm in diameter (Fig. 2g). Western blot analysis revealed that levels of mutant and wild-type ataxin-1 were similar (data not shown), suggesting that the difference in nuclear distribution between wild-type and mutant ataxin-1 was not due to a difference in expression levels. We conclude that the altered nuclear distribution of mutant ataxin-1 is due to the expanded polyglutamine tract.

There is increasing evidence that the nucleus is highly organized into functional domains¹¹, so we used molecular probes for selected nuclear components to characterize further the nuclear distribution of ataxin-1. Promyelocytic leukaemia protein (PML) is a nuclear matrix-associated protein and a component of PML oncogenic domains (PODs), which are also known as nuclear bodies^{4–7}. We used the antibodies to PML, SC35 (a component of spliceosomes)¹², p80-coilin (a component of coiled bodies)¹³ and BCL-6 (a transcription factor)¹⁴, in colocalization studies of SCA1-transfected COS-1 cells. Both wild-type and mutant ataxin-1 failed to colocalize with SC35, p80-coilin or BCL-6, and they had no effect on the

nuclear distribution of these proteins (Fig. 3d–l). However, in cells double-labelled with anti-ataxin-1 (ref. 8) and anti-PML antibodies⁴, the large nuclear structures characteristic of mutant ataxin-1 often sequestered PML and clearly altered its normal nuclear distribution (Fig. 3a–c). Although small structures of ataxin-1 occasionally colocalized with PML in COS cells transfected with wild-type ataxin-1, these are likely to happen by chance given the high number of structures formed by each protein per cell and the fact that most of the PML and ataxin-1 structures are distinct. Most importantly, expression of wild-type ataxin-1 in COS cells did not alter the normal distribution of PML. Overexpression of BCL-6 in COS cells also had no effect on PML nuclear distribution¹⁴, suggesting that the disruption of PML by mutant ataxin-1 is specific. Furthermore, the fact that the nuclear distribution of three other nuclear components was unaffected by the expression of mutant ataxin-1 suggests that the formation of the large structures containing ataxin-1 and PML is unlikely to be the result of a general loss of nuclear structural health and integrity.

Because PML is known to associate with the nuclear matrix, we investigated whether ataxin-1 is also a nuclear matrix-associated protein. We isolated the nuclear matrix from COS-1 cells transfected with ataxin-1 by means of cell lysis with a non-ionic detergent, nucleic acid digestion, and high salt extraction¹⁵. When the resultant matrix was stained with propidium iodide and anti-PML and anti-ataxin-1 antibodies, we detected PML and both wild-type (Fig. 4a) and mutant ataxin-1 (Fig. 4b). Ataxin-1 was also detected in western blot analysis of nuclear matrix preparations of transfected COS-7 cells (Fig. 4c). To demonstrate that ataxin-1 is a component of the nuclear matrix in cerebellar tissue, nuclear matrix preparations were performed on brain tissue from a wild-type SCA1 transgenic mouse. Western blot analysis of this material revealed the presence of ataxin-1 (Fig. 4d).

Our analyses of the subcellular localization of ataxin-1 in the Purkinje cells of SCA1 transgenic mice, neurons of SCA1 patients, and in transfected COS-1 cells have revealed that ataxin-1 localizes to nuclear compartments that associated with the nuclear matrix. In these systems the pattern of nuclear distribution differed for mutant and wild-type ataxin-1, as mutant ataxin-1 alone was associated with specific alterations of nuclear structure. These data suggest that the subcellular target of SCA1 pathogenesis is within the nucleus and involves the disruption of a nuclear function. Histological examination of neurons from individuals affected with two other polyglutamine disorders, Huntington disease and SCA3, revealed the presence of single large nuclear inclusions^{16–19} similar to those reported here. Furthermore, transgenic mice expressing a fragment of the Huntington's disease gene with an expanded number of glutamines have nuclear inclusions containing the protein huntingtin²⁰. Thus polyglutamine disorders involve pathogenic changes in the nucleus. Our observations that ataxin-1 associates with the nuclear matrix and that mutant ataxin-1 causes the specific redistribution of PML, another matrix-associated protein, may implicate a specific nuclear mechanism. The neuronal specificity of SCA1 pathogenesis is likely to be determined by the interaction of ataxin-1 with other nuclear proteins unique to those neurons affected by SCA1, such as the Purkinje-cell protein called leucine-rich acidic nuclear protein (LANP)²¹. □

Methods

Immunohistochemical analysis of cerebella. Anaesthetized mice were perfused with PBS-buffered formalin, fixed overnight, placed in PBS and stored at 4 °C. Vibratome sections ~50 μm thick were cut in the sagittal plane. Ataxin-1 epitopes were revealed by microwaving the sections three times for 20 s in 0.01 M urea^{8,22}. The sections were blocked for 1 h using 2% normal goat sera in PBS with 0.3% Triton X-100 and then incubated for 48 h in blocking solution with a 1:400 to 1:1,000 dilution of anti-ataxin 11750VII antibodies or a 1:4,000 to 1:10,000 dilution of anti-ataxin-1 11750VII (ref. 9) at 4 °C. Sections were washed four times for 20 min in PBS, incubated with blocking solution

Figure 2 Immunofluorescence of ataxin-1 in transfected COS-1 cells. **a**, The constructs. CMV, the CMV promoter; FL, the Flag epitope; numbers, amino-acid residues. **b, d, f**, The subcellular localization of wild-type ataxin-1[30]; **c, e, g**, the localization of mutant ataxin-1[82]. The percent of transfected cells with each depicted pattern it indicated. Images were generated by projecting a series of confocal Z scans that spanned the nucleus.

Figure 3 Confocal analysis of ataxin-1[82] and other nuclear proteins in COS-1 cells. **a**, Endogenous PML; **d**, endogenous SC35; **g**, endogenous p80-coilin; **j**, transfected BCL-6; **b**, **e**, **h**, **k**, mutant ataxin-1[82]; **c**, **f**, **i**, **l**, Merged images of PML and ataxin-1[82] (**c**), SC35 and ataxin-1[82] (**f**), p80-coilin and ataxin-1[82] (**i**), and BCL-6 and ataxin-1 (**l**). Scale bar, 5 μ m.

Figure 4 Association of ataxin-1 with the nuclear matrix. **a**, **b**, Ataxin-1[30] (**a**) and ataxin-1[82] (**b**) (green) in nuclear matrix from transfected COS-1 cells that were counterstained with propidium iodide (red) as a negative control, and anti-PML (blue), as a positive control. **c**, **d**, Western blots of subcellular fractions from ataxin-1[82] transfected COS-7 cells (**c**) and cerebella of ataxin-1[30] transgenic mice (**d**), probed with anti-ataxin-1 sera. The arrow indicates the ataxin-1 band. The subcellular fractions analysed in **c** and **d** include whole-cell protein extract; CSK extracts from whole cells (**c**) and isolated nuclei (**d**); DNase-soluble protein, DNase; $(\text{NH}_4)_2\text{SO}_4$ -soluble protein, $(\text{NH}_4)_2\text{SO}_4$; NaCl-soluble protein, 2 M NaCl; and protein remaining with the nuclear matrix. A lymphoblast (LB) lysate from an SCA1 patient, having alleles of 26 and 82 CAGs, was loaded into the last lane of **d**.

containing goat anti-rabbit polysera conjugated to Cy3 (Jackson ImmunoResearch) for 48 h, and washed four times for 20 min in PBS. Sections were mounted on slides with glycerol–gelatin containing 4 mg ml⁻¹ *n*-propylgalate and examined with a Bio-Rad MRC-1000 confocal microscope equipped with a krypton–argon laser. Human brain tissue from a SCA1 patient (29 and 82 CAG repeats) was obtained 2 h after death and immediately fixed. Paraffin sections (5 μ m) were used for immunostaining⁹. Antibodies used were an anti-ataxin-1 antiserum (11NQ) and a monoclonal anti-ubiquitin antibody (Novacastra). The 11NQ antiserum was raised to an N-terminal peptide (amino acids 164–197) of ataxin-1 conjugated through its N terminus to keyhole limpet haemocyanin. The 11NQ antiserum was raised in New Zealand rabbits by Cocalico Biologicals and characterized (A.S. & H.Y.Z., unpublished data).

Plasmids. The SCA1 cDNAs^{3,23} were subcloned into the *Eco*RI site of the eukaryotic expression vector pcDNA1Amp (Invitrogen). A Flag epitope was inserted downstream of the ATG codon in the SCA1[82] construct. Plasmid pVB140(EFBT), from T. Miki (Tokyo Medical and Dental University), contains a Myc-tagged BCL-6 cDNA under the control of the elongation factor promoter.

Immunohistochemical analysis of transfected COS-1 cells. The DEAE-dextran method of transfection²⁴ was used to transfect 1×10^6 COS-1 cells in 60-mm tissue-culture plates. Cells were transferred to sterile coverslips in 6-well tissue-culture plates 24 h post-transfection. Cells were fixed for 10 min in PBS plus 3.7% formaldehyde 48 h post-transfection, and permeabilized for 2 min with cold acetone. Coverslips were coated with 40 μ l of primary antibody

diluted in PBS. Either 1:100 dilutions of anti-flag M5 monoclonal antibodies (Kodak), anti-ataxin-1 11750V (1:100) or anti-ataxin-1 11750VII (1:200) polysera⁸ were used as primary antibodies. Anti-SC35 hybridoma supernatant¹² was used undiluted. Anti-PML (1:200) polysera⁵ and anti-PML monoclonal antibody 5E10 (1:10 dilution)⁴ were used. The anti-Myc Tag monoclonal antibody 9E10 (from V. Bardwell, University of Minnesota) was used to detect BCL-6. Anti-p80-coilin antibody^{25,26} was used to visualize coiled bodies. Goat anti-mouse conjugated to FITC (Caltag), goat anti-rabbit conjugated to lissamine rhodamine (Accurate Chemical and Scientific), goat-anti rabbit and goat anti-mouse conjugated to Cy2, Cy3 or Cy5 (Jackson ImmunoResearch) were used as secondary antibodies. Propidium iodide diluted in PBS (1 µg ml⁻¹) was used to stain nucleic acid. A subset of cells was also stained with bisbenzimidazole (0.001%) 10 min RT (Hoechst 33258 Sigma) as a transfection control. Images were collected and processed as described above.

Preparation of nuclear matrix. COS-1 cells seeded on coverslips were used for nuclear matrix preparations 48 h after transfection, as described previously¹⁵. Isolated matrices on coverslips were stained by immunofluorescence as described above. Nuclear matrix preparations from transgenic mice cerebella and COS-7 cells were carried out as described^{27–29}. Immunoblotting was performed as described⁸. Nuclei were extracted with SK buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 1.2 mM PMSF, 2 mM vanadyladenosine and protease inhibitors) for 3 min at 4 °C. Pelleted nuclei were suspended in digestion buffer (SCK plus 50 mM NaCl) with 400 U ml⁻¹ of RNase-free DNase I and digested for 50 min at 32 °C. Digested DNA was extracted with 0.25 M (NH₄)₂SO₄ and washed with 2 M NaCl in digestion buffer. For COS-7 cells, 2 × 10⁶ cells were extracted analogous to nuclear matrix preparation from transgenic mice, except that extractions were carried out on whole cells instead of isolated nuclei.

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The cerebellar leucine-rich acidic nuclear protein interacts with ataxin-1

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Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disorder characterized by ataxia, progressive motor deterioration, and loss of cerebellar Purkinje cells¹. SCA1 belongs to a growing group of neurodegenerative disorders caused by expansion of CAG repeats, which encode glutamine². Although the proteins containing these repeats are widely expressed, the neurodegeneration in SCA1 and other polyglutamine diseases selectively involves a few neuronal subtypes. The mechanism(s) underlying this neuronal specificity is unknown. Here we show that the cerebellar leucine-rich acidic nuclear protein (LANP)³ interacts with ataxin-1, the SCA1 gene product. LANP is expressed predominantly in Purkinje cells, the primary site of pathology in SCA1. The interaction between LANP and ataxin-1 is significantly stronger when the number of glutamines is increased. Immunofluorescence studies demonstrate that both LANP and ataxin-1 colocalize in nuclear matrix-associated subnuclear structures. The features of the interaction between ataxin-1 and LANP, their spatial and temporal patterns of expression, and the colocalization studies indicate that cerebellar LANP is involved in the pathogenesis of SCA1.

The protein ataxin-1, the SCA1 gene product, is found predominantly in the nucleus in neurons and the cytoplasm in peripheral tissues⁴. In SCA1, there is selective degeneration of cerebellar Purkinje cells and brainstem neurons¹ despite the wide expression pattern of ataxin-1. Cell-specific proteins may mediate the pathogenesis of SCA1 and the other polyglutamine neurodegenerative diseases.

To identify proteins that interact with ataxin-1, we performed a yeast two-hybrid genetic screen⁵ using a mutant human ataxin-1 containing 82 glutamines. Yeast expressing mutant ataxin-1 was transformed with a mouse brain cDNA library, and screening of approximately 1 × 10⁷ tryptophan and leucine auxotrophic transformants resulted in the identification of four positive clones. Sequence analysis of these clones identified partial cDNAs encoding 147 amino acids homologous to the rat leucine-rich acidic nuclear