F. Discussion.

F.1. Ataxin-3 is transported into the nucleus and associates with the nuclear matrix.

Previous immunohistochemical studies of human brain have localised the ataxin-3 protein, containing a polyglutamine sequence in the normal range (Q12-40), to both neuronal and non-neuronal cells in a predominantly cytoplasmic distribution. In contrast, in MJD diseased brains the protein was detected primarily in the nuclei of neurons (Paulson, *et al.* 1997), suggesting that an expanded glutamine repeat is responsible for the translocation of ataxin-3 into the nucleus. To determine whether a full-length ataxin-3 protein with a glutamine repeat in the normal range (Q_3KQ_{22}) is transported into the nucleus, COS-7 as well as neuroblastoma cells were examined by immunofluorescence microscopy, CLSM and subcellular fractionations. Using the affinity-purified anti-ataxin-3 antibodies NT1 or CT1 (directed against the N- and C-terminal portion of ataxin-3), the ataxin-3 protein was predominantly detected in the nucleus in untransfected mammalian cell lines (Fig. 7 C and Fig. 8 A, B), indicating that an elongated glutamine sequence is not necessary for the protein to be transported into the nucleus. In comparison, in COS-7 cells that have been transfected, a nuclear as well as cytoplasmic staining was detected (Fig. 7 A, B and Fig. 9) which is in agreement with the results obtained by Paulson, *et al.* (1997) who found for kidney epithelial 293T cells that the expression of the full-length ataxin-3 protein with a normal (Q27) or an expanded glutamine tract (Q78) resulted in a diffuse cytoplasmic staining. On the other hand, a truncated ataxin-3 protein with an expanded glutamine repeat did not distribute diffusely within the cytoplasm of the 293T cells, but rather localised to distinct subcellular structures in the cytoplasm and also in the nucleus, indicating that it is mainly the truncated ataxin-3 protein with an elongated glutamine sequence which translocates to the nucleus, where it eventually forms microaggregates

(Paulson, *et al.* 1997). In addition, evidence was presented that full-length ataxin-3 protein with a glutamine repeat in the normal range (Q12-40) is recruited by the truncated form of ataxin-3 into the nuclear aggregates. However, my results obtained with the untransfected COS-7 and neuroblastoma cells indicate that neither a truncation nor an expanded glutamine sequence is a prerequisite for the nuclear transport. These data were recently confirmed in an other cell model by Evert *et al.* (1999) using a stable ataxin-3 rat mesencephalic cell line (CSM14.1). The expression of expanded and non-expanded ataxin-3 protein was detected in both, nuclear and cytoplasmic compartments whereas the endogenous rat ataxin-3 protein showed a strong nuclear staining.

It has been shown recently for ataxin-1 that both the wild-type and mutated protein, containing glutamine repeats in the normal (Q30) or pathological range (Q82), respectively, are localised in the nucleus of cerebellar Purkinje cells or transfected COS-1 cells, indicating that this disease protein is transported into the nucleus independently of the presence of an elongated glutamine repeat (Skinner, *et al.* 1997). Furthermore, it was demonstrated that both the normal and the mutated ataxin-1 protein associate with the nuclear matrix. In my studies a strong fluorescent staining was detected when *in situ* matrix preparations or purified nuclei of neuroblastoma cells were examined by CLSM (Fig. 8 C, D, E and F), indicating that ataxin-3 similar to ataxin-1, is associated with the nuclear matrix. This has been demonstrated very recently also for ataxin-7 protein (SCA7) (Kaytor, *et al.* 1999).

To explore whether ataxin-3 contains a nuclear localisation signal (NLS), database searches were performed using the PSORT program (Nakai, 1991; Nakai, *et al.* 1992). Fig. 13 shows that there is a potential NLS and two casein kinase II sites (CK-II) immediately upstream of the polyglutamine sequence. It has been demonstrated that proteins that harbour a NLS very often also contain CK-II sites at a distance of approximately 10-30 amino acid residues from the NLS (Rihs, *et al.* 1991). While the NLS determines the specificity of

the nuclear transport, the CK-II site determines the rate of the transport. To test the hypothesis that this putative NLS might be involved in the translocation of ataxin-3 protein from the cytoplasm into the nucleus, I have mutagenized three of the four basic amino acids in the NLS domain. The transient expression of the mutated ataxin-3 protein, lacking in the NLS, did not show any differences in the subcellular localisation in comparison with the wild-type. From this result it would appear that the NLS does not play a critical role in the translocation of the ataxin-3 protein from the cytoplasm into the nucleus in COS-7 cells and that the localisation is more likely mediated by the size of the protein or by the presence of cell specific transport factors (Görlich, 1997). These hypothesis could also explain the different localisations of the wild-type ataxin-3 protein in different cell lines.

F.2. Aggregates.

Machado-Joseph disease is characterised by an expansion of CAG repeats in the *MJD1* gene encoding polyglutamine. The genetic and molecular features of the disease suggest a toxic gain of function of the ataxin-3 protein as probable mechanism of pathology. Similar to the other seven known neurodegenerative disorders caused by CAG expansions (HD, SCA1, SCA2, SCA6, SCA7, SBMA, DRPLA), MJD shows an inverse correlation between the length of the glutamine repeat and the age of onset of the clinical symptoms. This phenomenon clearly indicates that the number of glutamines is strikingly related to the cause of the disease. To give an explanation for this correlation, Max Perutz proposed the " polar zipper " theory (Perutz, 1994). According with this theory, the glutamine repeats self-associate, *via* hydrogen bonds, into large protein aggregates. Furthermore, thermodynamic considerations suggest that the elongation of the number of glutamines above a critical length (~41) results in a change of conformation of the glutamine chains from a random coil to a stable hairpin structure (Perutz, 1996; Trottier, *et al*. 1995). The hairpins then could self-assemble into a stable b-sheet structure. Other b-

sheet proteins such as APP and PrP^{sc} have been demonstrated as causative agents in Alzheimer`s and prion diseases, respectively, and both proteins have been shown to form proteinase-resistant aggregates (Hilbich, *et al.* 1991; Jarrett, *et al.* 1993). Using a GST-huntingtin fusion protein with polyglutamines in the pathological range, Scherzinger, *et al.* (1997) showed the *in vitro* formation of insoluble amyloid-like fibrils, similar to those found in Alzheimer`s disease and in prion rods (Prusiner, 1998). Digestion of the GST-huntingtin fusion proteins with factor Xa and trypsin clearly contributed to the formation of the polyglutamine-containing protein aggregates. These studies demonstrated that the formation of insoluble aggregates is not only dependent on the length of the repeats but also on the size of the fusion proteins. Moreover, other *in vitro* studies (Wellington, *et al.* 1998; Goldberg, *et al.* 1996) suggested the possibility that proteins containing polyglutamine expansions are substrates for caspase cleavage. The truncation of polyglutamine-containing proteins may promote the formation of insoluble protein aggregates *in vivo.* It may be that only truncated polyQ-containing protein is selectively accumulated in the nucleus and causes the selective neuronal cell loss (Martindale, *et al.* 1998; Schmidt, *et al.* 1998). Recently it has been proposed that the insoluble protein aggregates in HD patients consist of an N-terminal huntingtin fragment, but not of the full-length huntingtin protein (DiFiglia, *et al.* 1997). Furthermore, Paulson, *et al.* (1997) have demonstrated that the full-length ataxin-3 protein with a glutamine repeat in the pathological range does not form insoluble aggregates *in vitro,* whereas a truncated ataxin-3 protein with elongated glutamine repeat was able to form aggregates. My studies with the GST-ataxin-3 fusion proteins, containing polyglutamine tracts in the normal and pathological range, suggest that a certain number of glutamines are necessary for the formation of fibrillar aggregates. According to the result obtained with the cellulose acetate filter assay (CAFA), the critical length of the polyglutamine stretch for the formation of insoluble aggregates is 42 when the fusion protein was digested with trypsin. This result is in agreement with a change in conformation of the

polyglutamine stretch from a random coil to a hairpin structure as proposed by Perutz (Perutz, 1994). Moreover, the morphology of the ataxin-3 aggregates was similar to that of huntingtin aggregates (Scherzinger, *et al.* 1997). The number of glutamines required to observe aggregates increased to 64 when the entire ataxin-3 fusion protein was analysed. This indicates that the full-length ataxin-3 protein, is significantly more soluble than a truncated ataxin-3 fragment containing the polyglutamine stretch and demonstrate that the degree of aggregation is dependent also on the size of the amino acid sequence outside the polyglutamine domain. This data are in good agreement with the pathological threshold (Q55-84) (Lunkes, *et al.* 1997; Chai *et al.* 1999) and with the results obtained by Scherzinger, *et al.* (1997). Also in this case a GST-huntingtin fusion protein was more soluble than a truncated huntingtin protein with the same number of glutamines.

In cell culture systems the formation of polyglutamine aggregates seems to be more complicated and other factors must influence this phenomenon. Expression studies in COS-7 cells revealed that only ataxin-3 protein with more than 131 glutamines self-assembled into insoluble aggregates. This indicates that the level of expression as well as the *in vivo* environment plays a critical role in the formation of the aggregates. It is known that the kinetics of protein aggregation depends on protein concentration (Jarrett, *et al.* 1993; Scherzinger, *et al.* 1999). For instance, my analysis of a MJD lymphoblastoid cell line expressing a mutated ataxin-3 protein by immunofluorescence microscopy and by the cellulose acetate filter assay (CAFA) did not reveal the presence of polyglutamine aggregates. This could be due to different reasons. One of them is the fact that the lymphocytes are blood cells which divide frequently. The ratio between the level of protein expression and the life-time of the cells did not seem to permit the accumulation of aggregates. Moreover, the finding that only cells of certain tissues, expressing the mutated ataxin-3 protein, form aggregates and go to apoptosis, suggests that other cell-specific factors are involved *in vivo* in the formation of polyglutamine aggregates. Such factors could be proteins with a restricted pattern of expression in

specific brain regions or in particular tissues. For instance Sittler, *et al.* (1998) demonstrated that the SH3GL3 protein promotes the formation of polyglutamine-containing huntingtin aggregates in transfected COS-7 cells. This protein is selectively expressed in brain and in testis and interacts with the proline rich region of the exon 1 of huntingtin. In other studies regarding SCA1, Metilla, *et al.* (1997) discovered a selective interaction between LANP, a cerebellar leucine-rich acidic nuclear protein, with ataxin-1. This protein is selectively expressed in Purkinje cell nuclei, the primary pathological target in SCA1. Moreover, it has been shown that this interaction was stronger with longer glutamine repeats (82 *vs.* 30 Glu). Other polyglutamine binding proteins such as the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Burke, *et al.* 1996; Koshy, *et al.* 1996), the huntingtin associated protein 1 (HAP-1) (Li, *et al.* 1995), the huntingtin interacting protein 1 (HIP-1) (Wanker, *et al.* 1997; Kalchman, *et al.* 1997), the cystathionine b-synthase (Boutell, *et al.* 1998) and vimentin (Onodera, *et al.* 1997) have been discovered but none of those proteins has been shown to be involved in the pathomechanism. In case of the ataxin-3 protein no interacting protein has been described in the literature so far and two-hybrid studies performed in our laboratory did not reveal any potential protein candidate. On the other hand, Perez, *et al.* (1998) could demonstrate recently, that the TATA-binding protein (TBP) in human and the eyes absent protein (EYA) in *Drosophyla* are recruited into the ataxin-3 polyglutamine aggregates *in vivo.* Both proteins contain a glutamine repeat in the normal range. Therefore, they proposed that functional proteins containing a glutamine repeat in the normal range could be recruited into the glutamine aggregates and that the recruitment could contribute to the pathology. Moreover, in co-expression studies they also showed that both, normal and expanded full-length ataxin-3 protein are recruited into protein aggregates formed by a phatological fragment of the ataxin-3 protein (HA-Q78) or by the ataxin-1 protein (SCA1) containing an elongated glutamine repeat. Skinner, *et al.* (1997) showed that the ataxin-1 protein, which is transported into the nucleus, is associated with the nuclear matrix. In this

study I demonstrated by immunofluorescent microscopy that also ataxin-3 is present in the nuclear matrix (Tait, *et al.* 1998). These data suggests that the binding of ataxin-1 and ataxin-3 protein to a glutamine repeat in the pathological range could alter the structure of the nuclear matrix and therefore contribute to the pathology of these neurodegenerative diseases. In fact, Chai *et al.* (1999) demonstrated that cells, expressing a nuclear targeted mutant ataxin-3 [NLS-ataxin-3(Q78)], form nuclear inclusions which co-localise to the PML oncogenic domain (PODs). This domain is known to be part of the nuclear matrix and be altered by ataxin-1 inclusions (Zuber, *et al.* 1995; Skinner, *et al.* 1997). [Very recently the co-localisation with PODs has been demonstrated also for ataxin-7 protein (SCA7) (Kaytor et al. 1999)]. Although additional experiments will be necessary to prove whether the aggregation of ataxin-3 in the nuclei of neuronal cells causes the Machado-Joseph disease, the data presented in this work indicate that the formation of insoluble ataxin-3 protein aggregates could be a key event in the pathomechanism of this disorder. Therefore in the future a detailed analysis of the aggregates found in neuronal cells has to be performed.