Molecular biology of Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is the most common inherited human muscle disease and among the most debilitating. In recent investigations the locus of the gene in the Xp21 region of the X chromosome has been defined, the DMD gene has been cloned, and its protein product 'dystrophin' has been identified and partially characterized. The analysis of patterns of DMD gene and dystrophin defects in DMD and related disorders has provided insight into the molecular pathogenesis of differing clinical phenotypes of DMD. These studies have also identified two animal models homologous to human DMD. This review outlines these recent studies and their implications for understanding the molecular biology of DMD.

Duchenne muscular dystrophy (DMD) is a degenerative disorder of skeletal and cardiac muscle[1]. With rare exceptions, it occurs in males as an X-linked disease characterized by calf hypertrophy, predominantly proximal muscle weakness and an elevation in serum of muscle-specific enzymes such as creatine phosphokinase. The disease is evident in boys by 2 years of age and is usually fatal by the late 20s. Becker described a slowly progressive, X-linked myopathy whose clinical features suggested a close relationship with DMD. Becker muscular dystrophy (BMD) begins more insidiously than DMD and is compatible with survival into mid-life.

Pathologically, DMD and BMD muscle share common histological features, usually more severe in DMD[2]: muscle fiber necrosis, dissolution and phagocytosis; fiber hypertrophy and size variation and, particularly in DMD, extensive connective tissue proliferation (fibrosis). In addition, activated basophilic myoblasts and split or centrally nucleated fibers are thought to attest respectively to acute and chronic muscle regeneration.

The *DMD* gene

The cellular pathophysiology of DMD is not well understood, although extensively investigated. By contrast, the past five years have witnessed rapid progress in defining the molecular basis for this disease at both the DNA and protein levels. The chromosomal location of the *DMD* gene was first suggested to be the Xp21 region of the short arm of the X chromosome by cytogenetic analyses of rare females with Duchenne-like muscle diseases (reviewed in Ref. 3). In several of these patients it was observed that a portion of a non-sex chromosome (or autosome) was translocated to the X chromosome. Although the autosomes varied from case to case, in every instance the Xp21 region of the X chromosome was disrupted by the translocation. Fragments of the X chromosome near this Xp21 region were first cloned about five years ago[4,5].

The principal event in the molecular analysis of DMD was the cloning of the normal *DMD* gene. This was greatly facilitated by the identification of a male patient simultaneously afflicted with five disorders known to be linked to the X chromosome: DMD, mental retardation, retinitis pigmentosa, chronic granulomatous disease and the McLeod phenotype [high serum creatine kinase (CK); acanthocytosis of red blood cells][6]. The expectation that these disorders arose from a single, large chromosomal deletion was confirmed by the observation that the Xp21 region was visibly deleted in the patient's X chromosomes. Seven random DNA fragments contained within this large deletion were obtained using a competitive hybridization protocol[7]. One of the cloned fragments, pERT87, was a particularly good candidate to reside within or near the *DMD* locus because it was specifically deleted from nearly 10% of patients with DMD. When the cloned region of the X chromosome adjacent to the pERT87 fragment was enlarged to approximately 200 000 base-pairs (bp) by chromosome walking[3,7], it became evident that specific chromosomal deletions that caused DMD could originate either immediately to the left of the pERT87 fragment and extend to the right or immediately to the right and extend to the left. This observation was strong, classic genetic evidence that the pERT87 fragment of DNA actually resided within the *DMD* gene.

Like most chromosomal DNA, the 200 000 bp surrounding pERT87 consisted predominantly of introns (DNA fragments that are transcribed but not included in mature mRNA) with infrequent exons (regions of DNA that are both transcribed and translated into a protein product). To identify one or more *DMD* exons and thereby a fraction of the DMD protein-coding sequence, fragments from the 200 000 bp region from a variety of mammalian species were screened for homology to DNA (conserved sequences). One of these fragments, pERT87-25, was then used to identify a 14 kb cytoplasmic mRNA transcript present in fetal human skeletal muscle[8]. pERT87-25 was also used to isolate the complete coding sequence (cDNA) corresponding to the mRNA[9]. The complete cDNA included over 60 exons. Studies with these cDNA clones suggested that the *DMD* locus was phenomenally large, comprising 2000 kb or more[9,10]

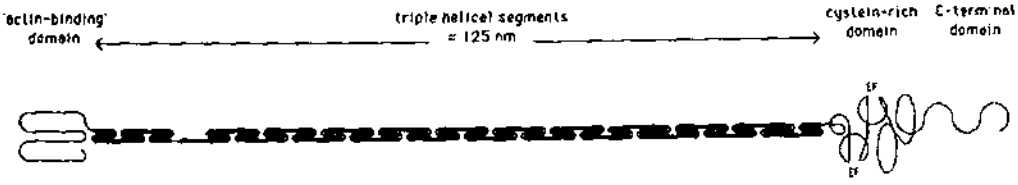


Fig. 1. *Model of the dystrophin molecule. Four possible domains of dystrophin are shown including speculative globular conformational loops in the actin-binding, cystein-rich and C-terminal domains. EF indicates two regions weakly homologous to EF-hand Ca^{2+}-binding loops in α-actinin.* (Taken, with permission, from Ref. 11.)

corresponding to 0.05% of the entire human genome or one-third of the genome of *E. coli*. Indeed, this very large size may be the reason for the unusually high mutation rate of the *DMD* gene[9].

Dystrophin, the DMD protein

Analysis of the predicted amino acid sequence from the cDNA indicated that the human fetal skeletal muscle form of the DMD protein consists of 3685 amino acids separable into four domains: (1) a 240 amino acid N-terminal domain showing homology to the putative actin-binding domain of α-actinin; (2) a large, rod-shaped domain composed of 25 triple-helical segments resembling repeat regions of spectrin; (3) a cysteine-rich domain partially homologous to the carboxy-terminus of α-actinin; and (4) a novel 420 amino acid sequence at the carboxy-terminal[11] (Figs 1 and 2). Sequence analysis does not reveal any transmembrane segments.

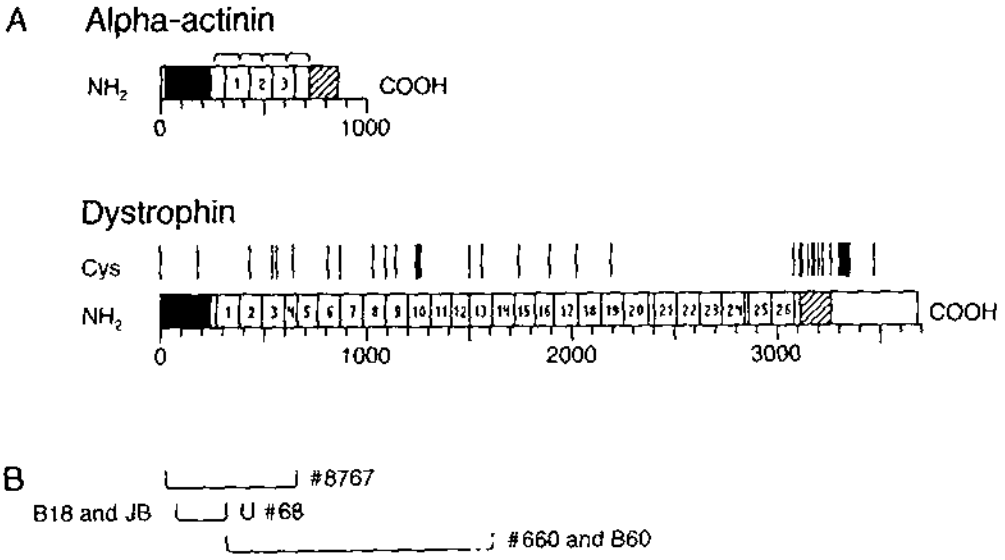


Fig. 2. *Comparison of domain organization of predicted amino acid structure of dystrophin and α-actinin.* **(A)** *Black box: putative actin-binding domain. Numbered boxes: helical repeat units. Four triple helical domains of α-actinin are shown by brackets above the repeat boxes. Streaked box: possible EF-hand Ca^{2+}-binding sites of α-actinin and weakly homologous segment of dystrophin. The scale in amino acids is given for both proteins.* **(B)** *Internal protein deletions in six patients with BMD are bracketed.* (Taken, with permission, from Ref. 11.)

The protein encoded by this locus, 'dystrophin', was first directly characterized using western blot analysis with polyclonal antibodies raised against bacterial trpE–dystrophin fusion peptides representing a total of 90 kDa of the 400 kDa DMD protein[12]. Dystrophin is an inabundant protein (approximately 0.002% of striated muscle protein) of 427 kDa. Tissue studies indicate that it is expressed in apparently equal levels in all muscle cells (skeletal, cardiac, smooth muscle) and in brain[12,13]. Dystrophin is also detected in cell culture in myotubes[14,15] (but not myoblasts) and in neural cells[14], probably within neurons[16]. It is not expressed in non-muscle or non-neural tissues *in vivo* or *in vitro*.

Three methods have been used to study the subcellular localization of dystrophin. Biochemical fractionation of skeletal muscle reveals that dystrophin is abundant in membranes and in transverse tubular fractions[17]. With immunofluorescent staining, the protein has been detected in association with the plasma membrane[18–20]. Initial electron microscope studies[21] have localized dystrophin to the inner face of the sarcolemmal membrane and probably the transverse tubules; it may be arrayed in a periodic network.

The precise function of dystrophin is unknown, but its similarity to spectrin and α-actinin, its extensive rod-shaped domain, and results of localization studies suggest it is a large, membrane cytoskeletal protein. Like spectrin in erythrocytes[22] and muscle[23], dystrophin may stabilize the muscle membrane. This role would accord well with evidence that the plasma membrane is abnormal in DMD muscle (see Ref. 24 for review; e.g. selective leakage of soluble muscle proteins into serum; the

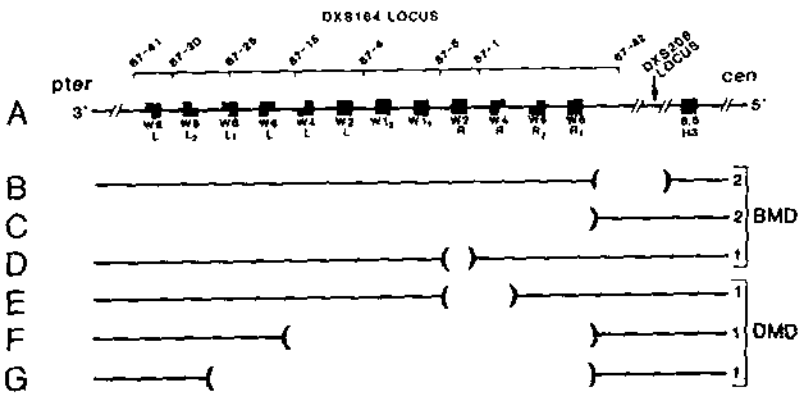


Fig. 3. *Relationship of DMD and BMD deletion breakpoints to exon : intron borders for genomic exons.* **(A)** *Exon : intron borders for 13 exons; 12 are within the pERT87 (DXS164) locus. Exons with jagged vertical borders (e.g. W9-L) break in the middle of a triplet codon; the number of base-pairs in these exons is not an integral multiple of three (e.g. 181 for W9-L). Exons with smooth vertical borders (e.g. W2-L) do not break within a triplet codon.* **(B–G)** *Lines showing patterns of deletions in patients with either BMD (B–D) or DMD (E–G). In* **(B)** *five exons (not shown) are deleted between W6-R1 and 8.5 H3. Analysis of exon : intron borders for W6-R1 and 8.5 H3 indicates that the deletion joins these exons without disrupting the open reading frame (ORF). This allows synthesis of dystrophin which will lack the deleted coding region of 232 amino acids or 26.6 kDa. In* **(C)** *multiple exons are missing from W6-R1 toward the 3′ end of the gene. Details of the 3′ end are not yet known but the breakpoint at the centromere (5′) end should preserve the ORF. A minimal deletion of 44.5 kDa is predicted in the resulting dystrophin molecule. In* **(D)** *deletion of exon W2-R of 6 kilobase-pairs does not disrupt the ORF and predicts a dystrophin lacking 4.45 kDa. In* **(E)** *the DMD patient lacks both exons W2-R and W4-R; deletion of the latter shifts the ORF and introduces a stop codon 141 base-pairs downstream which would terminate protein translation. The deletions in* **(F)** *and* **(G)** *shift the ORF and, respectively, introduce stop codons 10 and 43 base-pairs downstream. Note that the deletions in DMD may be smaller than those in BMD.* (Taken, with permission, from Ref. 26.)

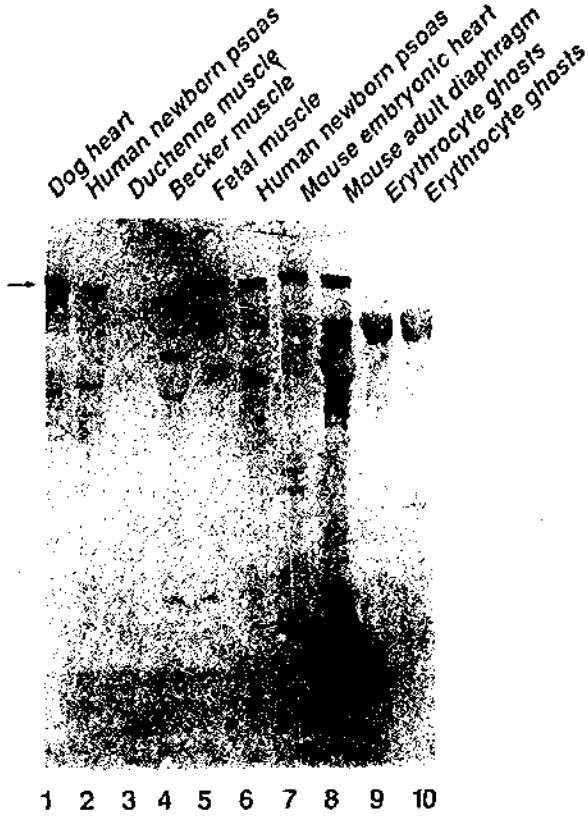


Fig. 4. *Western blots of dystrophin. The dystrophin molecule is detected by western blotting[12] in normal skeletal and cardiac muscle specimens (arrow). It is subnormal in size in the BMD case shown (lane 4); an atypical breakdown fragment often seen in BMD is evident in lane 4. By contrast, no dystrophin is seen in the Duchenne muscle (lane 3). The protein is not present in normal erythrocyte ghosts (lanes 9, 10).*

presence of pores or 'delta' lesions in otherwise unaffected muscle of DMD patients[25]). Dystrophin does not itself have an identified transmembrane region, so it is unclear whether these potential membrane abnormalities arise directly from lack of dystrophin or indirectly from displacement or dysfunction of proteins that link dystrophin to the membrane. In either instance, if the function of dystrophin is to preserve membrane integrity, a deficiency may be most critical in the most physically stressed muscle fibers. This correlation may explain the proximal distribution, age dependence and species dependence of muscle necrosis in the absence of dystrophin[16].

The molecular pathogenesis of DMD and BMD

These observations on the molecular biology of the DMD gene provide an important insight into the pathogenesis of DMD and BMD. As mentioned above, analysis of DNA from DMD patients with the initial pERT87 clones indicated that at least 7–8% of cases resulted from deletion mutations[7]. With the availability of the full DMD cDNA, the estimated deletion frequency was increased to 50% or more[9]. Interestingly, almost 80% of deletions are detected with two non-overlapping cDNA probes accounting for only 2 kilobase-pairs (kbp) (15%) of the total cDNA; moreover, nearly 40% of the deletion mutations apparently originate within a single intron. These features suggest that specific regions of genomic DNA within the *DMD* gene are in some way predisposed to deletions, either by virtue of sequence-specific rearrangement 'hot spots' or because the introns in question span exceptionally large distances of genomic DNA, resulting in an exceptionally large 'target' for mutation[9].

When the high frequency gene deletions underlying DMD and BMD was first appreciated, no clear correlation between deletion location or size and the severity of the resulting clinical disorder was observed. In some instances, severe DMD patients exhibited smaller deletions than mild BMD patients. Monaco and colleagues have recently provided insight into this apparent paradox[26] (Fig. 3). Detailed DNA sequence analysis in three DMD patients with intragenic deletions revealed that exclusion of the affected exons shifts the translational open reading frame of the resulting mRNA, thereby introducing a stop codon into the mRNA and terminating dystrophin synthesis (translation). By contrast, deletions in three BMD patients maintained the open reading frame of the resulting mRNA, potentially shortening but not precluding translation of the dystrophin molecule. Additional factors may also be important in the clinical diversity of *DMD* gene deletions. For example, in BMD there is no clear correlation between deletion size and the severity of the disease[11,26]. Of two patients with nearly identical, large deletions of helical repeats in domain 2, one required a wheelchair from the age of 16, whereas the other is ambulatory at 25. An understanding of these differences may provide insight into the function of specific sub-domains of the dystrophin molecule.

The availability of antibodies to dystrophin has allowed an analysis of DMD and related dystrophies at the protein level by western blotting (Figs 4 and 5). It appears that there is an excellent correlation between quantitative and qualitative abnormalities of dystrophin in muscle and the severity of the resulting clinical disorders[27]. In 35 of 38 patients with DMD, muscle dystrophin was either absent or marginally detectable. By contrast, in 12 of 18 BMD patients, dystrophin exhibited an altered molecular weight but was of nearly normal abundance. Dystrophin was normal in 38 of 40 patients with other unrelated neuromuscular disorders.

In this series, cases occasionally raised the possibility that defects within the *DMD* gene may produce clinical phenotypes not typical for DMD and BMD. For example, dystrophin blots characteristic of BMD were seen in a patient with the diagnosis of scapuloperoneal dystrophy and another with spinal muscular atrophy or limb-girdle dystrophy[27].

A small subset of DMD patients was found to synthesize dystrophin of normal size but markedly reduced quantity. These cases cannot have arisen simply from an aberrant stop codon, and so indicate that there may be multiple molecular mechanisms accounting for dystrophin deficiency (and dysfunction). Conceivably, the dystrophy in these instances may have arisen from defective dystrophin turnover (e.g. slowed translation or accelerated degradation of the defective dystrophin molecule) or some

defect in post-translational modification of the molecule. There is ample evidence that the expression of spectrin along the inner face of the red cell membrane is controlled at multiple levels involving both the turnover of the individual subunits and their post-translational modification[22]. These considerations may be of general importance in muscle pathology insofar as there is a precedent for the existence of mRNA with subnormal levels of protein in other genetic muscle disorders, such as McArdle's disease[28] or phosphoglycerate kinase deficiency[29].

Animal models of DMD

In 1984, Bulfield and colleagues[30] described an X-linked mutant mouse with elevated levels of plasma creatine kinase and histopathological findings compatible with muscular dystrophy. In affected 'mdx' mice, the muscle mRNA dystrophin transcript is apparently of normal size but subnormal (approximately 25%) abundance[13]; there is no corresponding dystrophin in the muscle[12]. Given the disease specificity of dystrophin deficiency in humans, it appears likely that this mdx mouse is a long-sought animal model of DMD. More recently, Cooper *et al.* have documented that an X-linked dog myopathy (CXMD) is also a DMD analogue[31]. In CXMD dystrophy, neither dystrophin nor its mRNA transcript are detected.

By contrast with canine and human DMD, the lack of dystrophin in the mdx mice is not lethal[30]; mdx mice survive normally without clinical disease. Neonatal mdx muscle is normal; muscle degeneration is not evident until three to five weeks postpartum. By nine weeks the major histopathological change is an abundance of central nuclei with considerably less active myonecrosis. The dense connective tissue proliferation characteristic of both DMD and CXMD muscle is not seen in mdx muscle. Why mdx mice are clinically normal despite the absence of dystrophin remains unexplained, although it has been suggested that the absence of extensive fibrosis may be a factor[12]. Other possible explanations may include the relative maturational delay in mouse muscle as compared with human and dog muscle or the smaller size of the mouse and its muscle fibers. It is anticipated that these animal models will prove important in understanding the basis for the malignancy of human DMD and in experimental approaches to pharmacotherapy of this disorder.

Unanswered questions and future research

Numerous unanswered questions will undoubtedly be addressed in the near future. Perhaps the most important will be the precise function of dystrophin. This will be critical in understanding the pathophysiology of the protein: how it prevents muscle necrosis, how its absence leads to proximal weakness and calf hypertrophy, exactly why the clinical courses of DMD and BMD differ, and whether other X-linked myopathies or cardiomyopathies are related to this gene. From a neurobiological perspective it is important to understand why 30% of DMD cases are mentally retarded[32]; available data do not suggest this is the result of mutation in a closely linked but distinct Xp21 gene. As noted above, the *DMD* gene may be expressed in neurons[16]; conceivably dystrophin or a related protein is critical in normal neural development, regeneration and/or function. Like the variations in muscle phenotype in DMD and BMD, the degree of intellectual impairment may depend in part on the nature of the dystrophin defect in each case. Finally, it is of urgent interest to study the regulation of expression of the *DMD* gene. Are there agents that enhance *DMD* gene activation and dystrophin synthesis? By which proteolytic pathways are normal and abnormal dystrophin degraded and can the degradation be retarded pharmacologically? Does the protein undergo post-translational modification and are its exons alternatively spliced in different muscle tissues? If recent progress in this field is any gauge, it is likely that initial answers to these questions will soon be found and conceivable that rational approaches to DMD therapy will evolve from this research, at least for those patients expressing some form of dystrophin.

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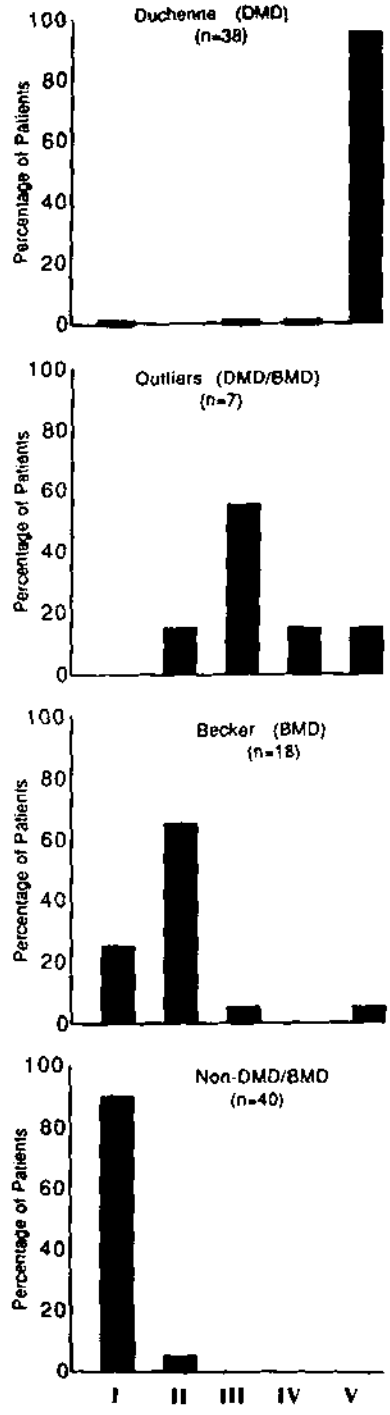


Fig. 5. *Biochemical phenotypes of dystrophin relative to clinical diagnoses. The histograms illustrate the percentage of patients corresponding to each dystrophin category as designated by Roman numerals: I, dystrophin of normal size and 60–100% of normal quantity; II, abnormal size, level 40–100% normal; III, normal size, quantity 3–60% normal; IV, abnormal size, level 3–40% normal; V, dystrophin undetectable. Outliers were patients whose clinical status was intermediate between DMD and BMD.* (Taken, with permission, from Ref. 27.)

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techniques

Organotypic cultures of neural tissue

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Organotypic cultures preserve the basic structural and connective organization of their tissue of origin. The high degree of neuronal differentiation, coupled with the organotypic location of neurons within a given network, permit morphological identification of types of neurons either in the living state or following histological staining. Easy access to nerve cells not only facilitates the use of standard electrophysiological techniques, but also makes these cultures amenable to patch-clamp methods. Furthermore, the successful demonstration of functional synaptic connections between co-cultured slices suggests that this technique offers a unique approach for studying synaptic transmission between remote brain areas.

Tissue culture methods

Since the pioneering experiments of Harrison in 1907[1], organotypic cultures have played an important role in elucidating mechanisms related to fiber outgrowth, myelin formation and development of synaptic networks. The general properties of these cultures have been summarized in a number of excellent reviews[2,3]. The term organotypic, originally introduced by Maximov, is employed to emphasize maintenance of characteristic properties unique to the tissue of origin[3].

The tissue to be cultured is separated from the rest of the brain by manual dissection using razor blades or scalpels, and then cut into approximately 400 µm thick slices by means of razor blades (explants) or by a mechanical tissue sectioner such as a McIlwain tissue chopper[4] (slice cultures). Individual slices are placed on glass coverslips and embedded on a suitable substrate like collagen or a plasma clot. The cultures are fed with a semi-synthetic medium consisting of a nutrient medium such as Eagle's, a balanced salt solution and serum in a concentration of 10–30%. Feeding schedules and survival times depend on the particular culture technique used; long-term survival for several weeks to months is a general feature of organotypic cultures.

The most common methods for preparing organotypic cultures of nervous tissue make use of either the Maximov assembly or the roller-tube technique. Cultures maintained in Maximov depression slide chambers as 'lying-drop' preparations[2,3] are highly differentiated and can be observed without removal from the culture assembly. Since they remain several cell layers thick, they are the method of choice for studies requiring the preservation of three-dimensional intercellular relationships and the frequent observation of the culture during the cultivation period.

Cultures prepared by the roller-tube technique are maintained on glass coverslips in plastic test tubes and slowly rotated to ensure proper aeration and feeding. These cultures[5] retain most of their original cytoarchitecture and attain a high degree of cellular differentiation, although the tissue flattens to monolayer or nearly monolayer thickness. Under these conditions, individual living neurons can easily be observed, thus allowing electrophysiological recording of morphologically identified neurons in all parts of a culture, not only in outgrowth areas.

Organotypic cultures are prepared from embryonic or neonatal tissue derived from various species. The age of the donor animals greatly influences the degree of tissue organization; in young tissue, cells tend to migrate and the original cytoarchitecture becomes partly distorted. On the other hand, special procedures (e.g. oxygen supply) have to be applied for the cultivation of tissue derived from older animals. Five- to seven-day-old rats provide a good compromise for the preparation of hippocampal, septal or hypothalamic cultures[5].

Although the basic principles of the culturing techniques are straightforward, organotypic cultures are not easy to prepare. Dissection of discrete brain regions from young animals presumes detailed knowledge of brain anatomy[6]. Moreover, handling of the freshly isolated tissue is quite delicate and requires a great deal of experimental skill. Finally, cultures grown in sealed Maximov assemblies need frequent and time-consuming feeding schedules.