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Methylation and Expression of the Myo D1 Determination Gene

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Phil. Trans. R. Soc. Lond. B 1990 **326**, 277-284
doi: 10.1098/rstb.1990.0011

Phil. Trans. R. Soc. Lond. B **326**, 277–284 (1990)
Printed in Great Britain

Methylation and expression of the *Myo* D1 determination gene

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Mouse embryo cells induced to differentiate with the demethylating agent 5-azacytidine represent an excellent model system to investigate the molecular control of development. Clonal derivatives of 10T1/2 cells that have become determined to the myogenic or adipogenic lineages can be isolated from the multipotential parental line after drug treatment. These determined derivatives can be cultured indefinitely and will differentiate into end-stage phenotypes on appropriate stimulation. A gene called *Myo* D1, recently isolated from such a myoblast line, will confer myogenesis when expressed in 10T1/2 or other cell types (Davis *et al.* 1987). The cDNA for *Myo* D1 contains a large number of CpG sequences and the gene is relatively methylated in 10T1/2 cells and an adipocyte derivative, but is demethylated in myogenic derivatives. *Myo* D1 may therefore be subject to methylation control *in vitro*. On the other hand, preliminary observations suggest that *Myo* D1 is not methylated at CCGG sites *in vivo* so that a *de novo* methylation event may have occurred *in vitro*. These observations may have significance in the establishment of immortal cell lines and tumours.

Introduction

The development of the differentiated tissues of a multicellular organism from a single fertilized egg proceeds in a highly ordered, stepwise fashion. During development, cells become progressively restricted with regard to their developmental options and become determined to particular cell lineages that subsequently differentiate into highly specialized cell types. Some of the most fascinating questions in developmental biology concern the molecular mechanisms responsible for this restriction of cellular phenotype and specialization of function.

The process of cell specialization can often be separated into two steps, called determination and differentiation, which have recently become amenable to molecular analysis. Cells with more than one developmental option initially become determined to a particular phenotype. The determined state can be inherited and cells within this state have the option of dividing to self-renew or of differentiating into the final phenotype characteristic of that particular cell type. For example, cells determined to a myogenic lineage are capable of dividing as myoblasts or of differentiating into end-stage myotubes upon receiving an appropriate extracellular signal (Konieczny & Emerson 1984). Much of our knowledge concerning the activity and structure of tissue-specific genes comes from an analysis of this latter process of end-stage specialization. Thus the structure and function of tissue-specific genes such as muscle myosin, creatine phosphokinase, tropomyosin, the acetylcholine receptor and other proteins responsible for the assembly and function of the contractile apparatus in muscle cells are known. On the other hand, little is known about molecular mechanisms responsible for the determination process, which is of great importance during development of the organism.

Recently, several experimental systems have been developed to approach the problem of

studying determination. One of the key requirements for a molecular analysis of this process, is the isolation of sufficient numbers of pure cell populations with well defined developmental options. This has been difficult to achieve in the complex mixture of cells and tissues that constitutes an organism. However, recent experiments making use of culture systems have allowed for the isolation of pure clonal populations of cells suitable for such an approach. This article will therefore focus on one such system that can be used for the molecular and cellular analysis of mesenchymal differentiation.

DNA methylation appears to play a significant role in cellular memory and it has long been thought that this information coding system could function in restricting cells to particular developmental pathways (Holliday & Pugh 1975; Riggs 1975). This review will examine the evidence that the methylation of cytosine residues within developmentally important genes may play a role in cellular determination.

5-Azacytidine

The nucleoside analogue 5-azacytidine (5-Aza-CR) was synthesized for use as a cancer chemotherapeutic agent. It contains an extra nitrogen in the pyrimidine ring, which leads to instability in aqueous solutions (Veselý & Čihák 1978). The drug is metabolized by uridine-cytidine kinase to the monophosphate and, in treated cells, is incorporated into both the RNA and DNA. The analogue is toxic to mammalian cells and has found limited use as an experimental chemotherapeutic agent. Many of the biological properties of 5-aza-CR are shared by the deoxy analogue 5-aza-2′-deoxycytidine (5-Aza-CdR), which is metabolized initially by deoxycytidine kinase to the monophosphate, and is incorporated only into the DNA of treated cells.

The presence of the extra nitrogen atom in the 5 position of the pyrimidine ring makes 5-azacytosine highly refractory to enzymatic methylation within nucleic acids (Friedman 1979; Jones & Taylor 1980; Jones 1985*a*, *b*). Indeed, the 5-azacytosine moiety is the most powerful inhibitor of methylation of both RNA and DNA. Azanucleosides act to inhibit methyltransferase enzymes apparently by the formation of a tight covalent bond between the enzyme and the six position of the triazine ring. Santi *et al.* (1983) have proposed that the drug acts as a suicide inhibitor of the methyltransferase in a manner analogous to the inhibition of the thymidylate synthase enzyme by 5-fluoro-2′-deoxyuridine monophosphate. Thus 5-Aza-CR and 5-Aza-CdR have found considerable experimental use, not only in the investigation of the relation between DNA methylation and gene expression but also in understanding the fundamental biochemistry of methyltransferase action.

Changes in differentiation elicited by 5-azacytidine

5-Aza-CR has marked effects on the stability of the differentiated state of cultured cells. Treatment of the 10T1/2 line of mouse embryo cells with micromolar concentrations of the analogue for brief time periods induces the formation of cells with the morphological appearance of muscle, fat and cartilage (Constantinides *et al.* 1977, 1978; Taylor & Jones 1979). The differentiated phenotypes have all of the biochemical and functional properties expected of cells cultured from these tissue types. For example, the muscle cells show the expression of myosin ATPase activity, the acetylcholine receptor (Constantinides *et al.* 1978)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

and the muscle form of creatine phosphokinase (Liu *et al.* 1986). The myotubes are striated and are functional as they can twitch either spontaneously or in response to acetylcholine. The adipose cells express all of the appropriate enzymes for fatty acid biosynthesis. The cartilage phenotype is also authentic and the cells produce type II collagen and the appropriate glycosaminoglycan extracellular matrix material (Taylor & Jones 1979).

The new phenotypes induced by 5-aza-CR therefore appear to be biochemically normal and are induced in the cells after brief exposures to the analogue in a process requiring cell division after drug treatment (Taylor & Jones 1982). In addition to inducing these marked changes in phenotype, the drug has shown itself to be remarkably effective in activating the expression of suppressed genetic information within other cell types (Jones 1985*a*, *b*). Many genes that are present in a quiescent state can be activated at very high frequencies after brief exposures to the analogue suggesting that they have become inactivated in culture by a methylation associated mechanism. Because these systems have been reviewed in the past (Jones 1985*b*; see also Holliday, this symposium) they will not be re-examined here.

Isolation of determined cell clones

A benefit of the use of 5-aza-CR to elicit changes in cellular development is that it allows for the isolation of cells with new biological traits that are expressed in a stable manner after drug treatment (figure 1). Clones can be isolated from cultures of 10T1/2 cells exposed to the drug that have the morphological appearance of myoblasts, adipoblasts, or chondroblasts (Konieczny & Emerson 1984; Harrington & Jones 1988). These derivatives can subsequently be recloned to obtain pure populations of cells with phenotypes distinct from the parent

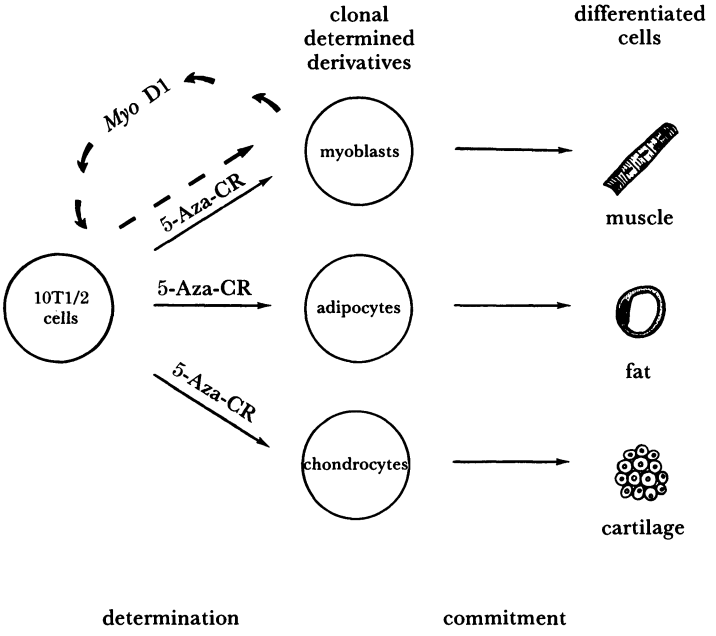

Figure 1. New pathways of differentiation induced in 10T1/2 cells by 5-azacytidine. Treatment of 10T1/2 cells with 5-aza-CR allows the isolation of myoblasts, adipoblasts or chondroblasts determined to their specific lineages. These cells can subsequently differentiate into end-stage phenotypes on an appropriate stimulus. The *Myo* D1 gene was isolated from determined myoblasts by Davis *et al.* (1987) and can confer myogenic potential on untreated 10T1/2 cells. (Modified from Harrington & Jones (1988) and reproduced with permission.)

10T1/2 cells. Several investigators have isolated cells with the phenotypic properties of myoblasts (Konieczny & Emerson 1984; Lassar *et al.* 1986; Liu *et al.* 1986) or adipoblasts (Chapman *et al.* 1984). These derivatives have varying degrees of stability and express the complete repertoire of genes associated with the authentic cellular phenotype. The end-stage differentiation of the cells requires a second signal, which is normally confluence within the culture. Thus myoblast cell lines can be grown for many passages and will reproducibly fuse to form multinucleated tubular myotubes when the culture reaches a monolayer or when growth factors are depleted. Similarly, adipoblast lines form fat droplets after reaching confluency.

The 10T1/2 cell may therefore be regarded as being a multipotential cell type whose developmental options have become defined following 5-aza-CR treatment. The phenotypes obtained after 5-aza-CR treatment appear to be mutually exclusive (figures 2 and 3). Cells that have the morphological characteristics of muscle cells express the δ subunit of the acetylcholine receptor (figure 2) but do not express the adipsin and *AP*2 genes (figure 3), which are specific for the adipocyte phenotype (Spiegelman 1988). On the other hand, cells selected for the adipocyte phenotype are restricted to this phenotype and express the *AP*2 and adipsin genes (figure 3) but do not express the acetylcholine receptor mRNA associated with the muscle phenotype (figure 2). None of these specialized products are expressed by 10T1/2 cells.

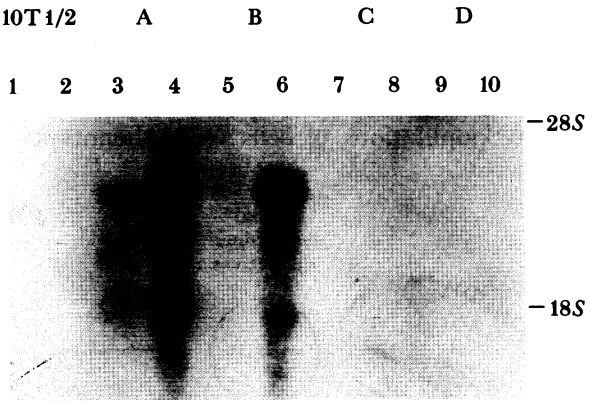

FIGURE 2. Expression of a muscle marker by determined 10T1/2 derivatives. Northern-blot analysis of the δ subunit of the acetylcholine receptor synthesized by growing (odd-numbered lanes) and confluent (even-numbered lanes) 10T1/2 cells, 3 myogenic derivatives (A, B and C) and fat cell derivative (D). These genes are expressed strongly particularly after confluence in the myogenic lines A and B and weakly in the unstable myogenic line (C). The muscle marker is not detectable in 10T1/2 cells or the fat cell line, D. Size markers are for 28*S* and 18*S* ribosomal RNA.

Figure 1 suggests that the changes in phenotype elicited by 5-aza-CR are single-step events in which the new phenotypes are induced directly and independently from the parental cells. However, it is still not clear whether there might be an intermediate cell type formed after 5-aza-CR treatment that subsequently changes into the three new lineages (Harrington & Jones 1988; Harrington *et al.* 1989). There is some evidence for the existence of such intermediates and we have isolated a cell line capable of differentiating into muscle fat or chondrocytes in the absence of any further 5-aza-CR treatment (Harrington *et al.* 1989). The existence of bipotential cells calls into question the hypothesis put forward in figure 1, and alternative routes for the formation of the new lineages may be possible.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

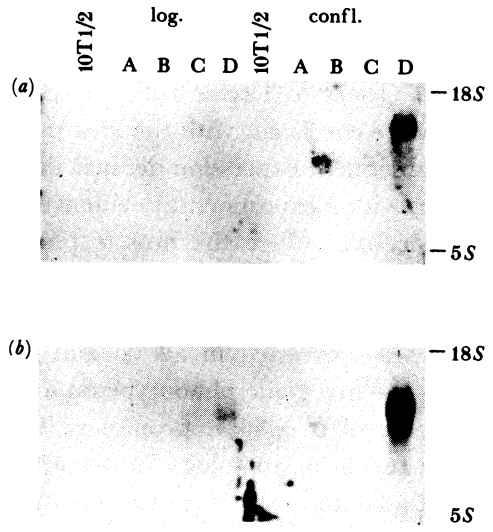

FIGURE 3. Expression of adipocyte markers by determined 10T1/2 derivatives. Northern-blot analysis of RNA extracted from logarithmic phase (log.) or confluent (confl.) cultures showing expression of the (*a*) adipsin and (*b*) *AP2* genes in the adipocyte derivative D but not in the parent 10T1/2 cells or myogenic derivatives A, B or C. Size markers are for 18*S* and 5*S* ribosomal RNA.

Irrespective of the exact route by which these new phenotypes are formed, the 10T1/2 cell system primed to differentiate with 5-aza-CR is an ideal one for studying the genes responsible for cellular determination. Comparison of the multipotential 10T1/2 cell with the determined cellular derivatives shown in figure 1 allows for the isolation of new genes that are activated as a result of drug treatment and that cause the changes in cellular potential. The availability of these clonal cell populations has lent itself to a molecular analysis that would have been extremely difficult in the complex mixture of cells constituting a multicellular organism.

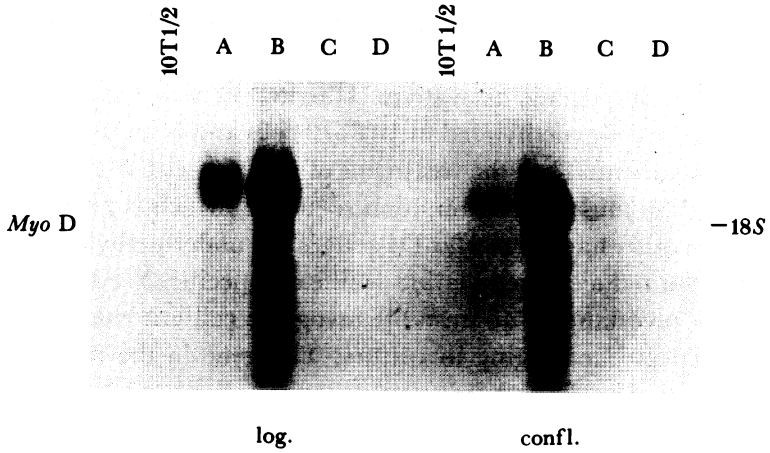

FIGURE 4. Expression of the *Myo* D1 gene in logarithmic and confluent cultures of myogenic cells (A, B and C) but not in 10T1/2 and an adipogenic derivatives (D). Northern-blot analysis of RNA extracted from growing or confluent cultures probed with *Myo* D1. Size marker for 18*S* ribosomal RNA.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Isolation of the *Myo* D1 determination gene

All of our initial observations (Jones & Taylor 1980; Taylor & Jones 1982) and those of Konieczny & Emerson (1984) were consistent with the idea that 5-aza-CR activated one or a small number of genes whose subsequent expression defined the new phenotypes. The elegant studies of Lassar *et al.* (1986) provided experimental evidence for this by showing that DNA extracted from myogenic cells could induce the muscle phenotype in recipient untreated 10T1/2 cells (figure 1). Subsequently, Davis *et al.* (1987) used the technique of subtractive DNA hybridization to isolate a gene from a myogenic 10T1/2 derivative that was not expressed in 10T1/2 cells but was expressed in all skeletal muscle cells tested. This gene, *Myo* D1, is capable of imposing the myogenic phenotype on a variety of cell types into which it was transfected under the control of a viral promoter. The *Myo* D1 gene is a nuclear phosphoprotein that contains a region of homology to the *myc* gene and also contains a zinc finger region. The expression of a 68 amino acid portion of *Myo* D1 containing a basic region and a region with similarity to the *myc* domain can convert 10T1/2 cells to muscle (Tapscott *et al.* 1988). Genetic analysis has mapped the *Myo* D1 gene to mouse chromosome 7 and human chromosome 11 (Tapscott *et al.* 1988). The gene product is located in the nucleus where it presumably functions as a DNA binding protein to control the expression of the subsets of genes necessary for the formation of a muscle cell. *Myo* D1 may therefore be a key member of a hierarchy of regulatory genes specifying mammalian development (Blau 1988).

Methylation of the *Myo* D1 gene

The cDNA for the *Myo* D1 determination gene (Davis *et al.* 1987) shows a very high concentration of CpG sequences and meets the criteria of a CpG island (Bird 1986; Gardiner-Garden & Fromner 1987). The genomic sequence has not yet been determined but preliminary studies in our laboratory suggest that the mouse gene is not very large and that the coding sequence is probably contained within approximately 3–4 kilobases (kb) of DNA.

We have used the cDNA probe to investigate the methylation status of the gene in 10T1/2 cells and in myogenic and adipogenic derivatives (Harrington *et al.* 1989). These studies have shown that the gene is relatively methylated in 10T1/2 cells and is unmethylated in myoblasts elicited by 5-aza-CR treatment. Thus the treatment of the cells with 5-aza-CR has resulted in the hypomethylation of the muscle determination sequence, which is presumably responsible for its activation. On the other hand, the *Myo* D1 gene is not undermethylated in an adipogenic cell line showing that the reduced methylation is lineage specific. We have also investigated *Myo* D1 methylation in revertants of an unstable myogenic cell line that have lost the ability to form muscle after repeated passaging in culture. The gene in the revertants has become remethylated and additional studies have demonstrated that further treatment with 5-aza-CR can reactivate the muscle phenotype. Thus all data are consistent with the idea that 5-aza-CR acts to induce the undermethylation of the determination gene in myogenic but not adipogenic cells and that the remethylation of the gene in revertants is associated with the loss of the myogenic phenotype.

In contrast to these results with cell lines in which the methylation status of the gene correlates both with expression and phenotype, preliminary analysis of the methylation of *Myo* D1 in a variety of tissues derived from adult mice shows that it is completely unmethylated

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

in all tissues tested (M. Wolkowicz & P. A. Jones, unpublished results). This is true even in tissues such as testis and brain that do not express *Myo* D1. The methylation of *Myo* D1 seen in cultured cells therefore appears to have occurred as an artefact of *in vitro* culture.

Determination genes may therefore become controlled differently in a culture environment relative to the animal. In this regard, many experiments have demonstrated dramatic changes in DNA methylation levels (Wilson & Jones 1983) and patterns (Shmookler-Reis & Goldstein 1982) in culture. Wise & Harris (1988) have shown that cytosolic thymidine kinase in V79 Chinese hamster cells can become inactivated by *de novo* methylation of the 5′ region under bromodeoxyuridine selection. The gene contains a CpG island in this region, so that although there is no evidence for the methylation of these islands in autosomal genes in animals (see Bird 1986; Gardiner-Garden & Frommer 1987), they can become *de novo* methylated in culture.

Discussion

The data are therefore consistent with the idea that the *Myo* D1 gene is not subject to methylation control *in vivo*, but is kept silent in the 10T1/2 system by unusual *de novo* methylation events. Previously, we examined overall methylation changes that occurred during the establishment of mouse cell lines in culture (Wilson & Jones 1983). These studies showed that after a rapid decrease in genomic 5-methylcytosine levels, an apparent re-methylation of DNA occurred in those cells that evolved into new immortal lines. It is conceivable therefore that determination genes such as *Myo* D1 become *de novo* methylated during the establishment of cell lines and that this contributes to cellular immortality.

Carcinogenesis is another process that may be influenced strongly by abnormal methylation events. It is well-known that carcinogens interact with the methylation machinery (Jones 1986) and that cancer cells have increased levels of methylase enzyme (Kautiainen & Jones 1986). Baylin *et al.* (1987) have found hot spots of hypermethylation in genes located on chromosome 11*p* in several human cancers. Thus lack of control in a cellular process, which is as powerful in extinguishing gene expression as DNA methylation, might have strong effects on cell longevity and transformation. The methylation of subsets of determination genes may be therefore important in the establishment of cellular immortality both in culture and in carcinogenesis.

This work was supported by grants CA 39913 and T32 CA 09569 from the National Cancer Institute.

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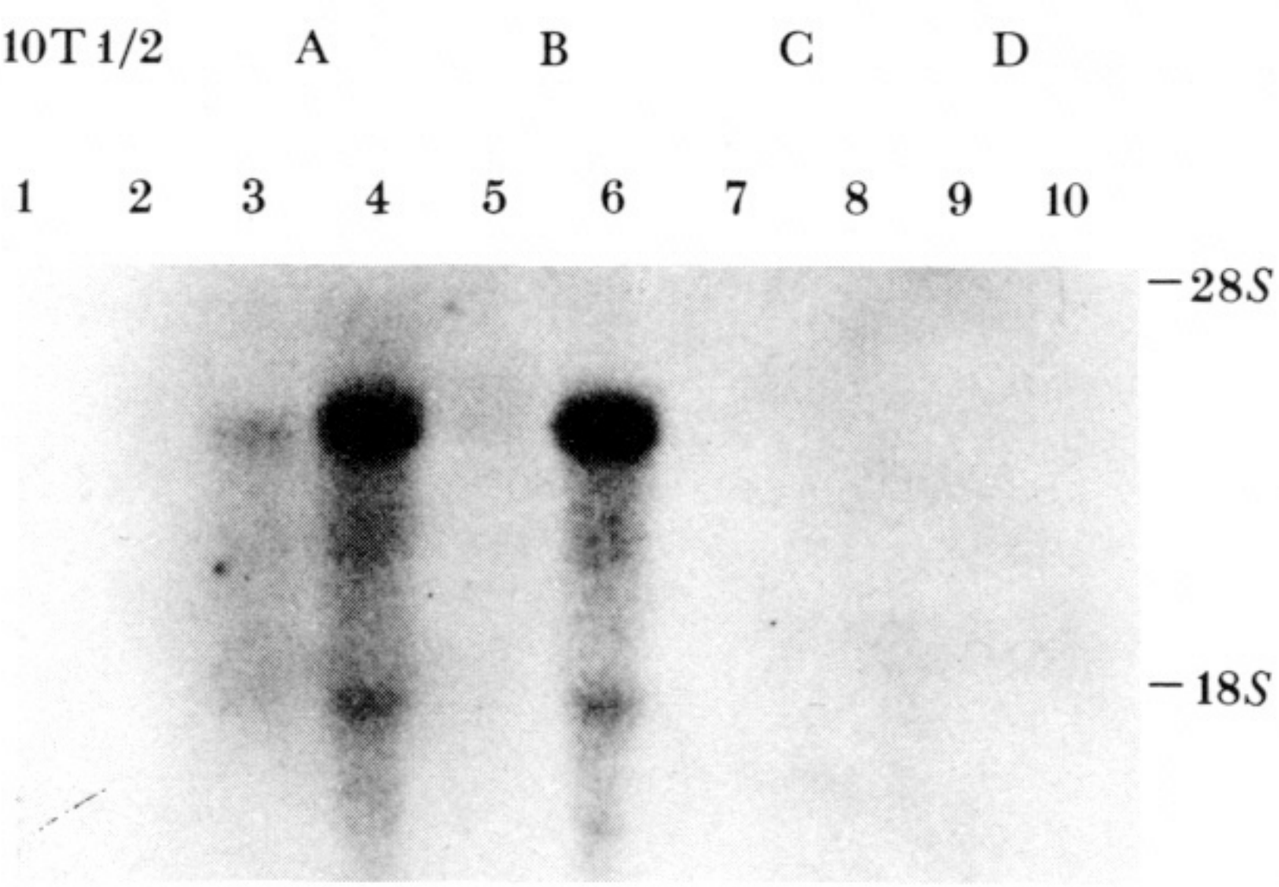

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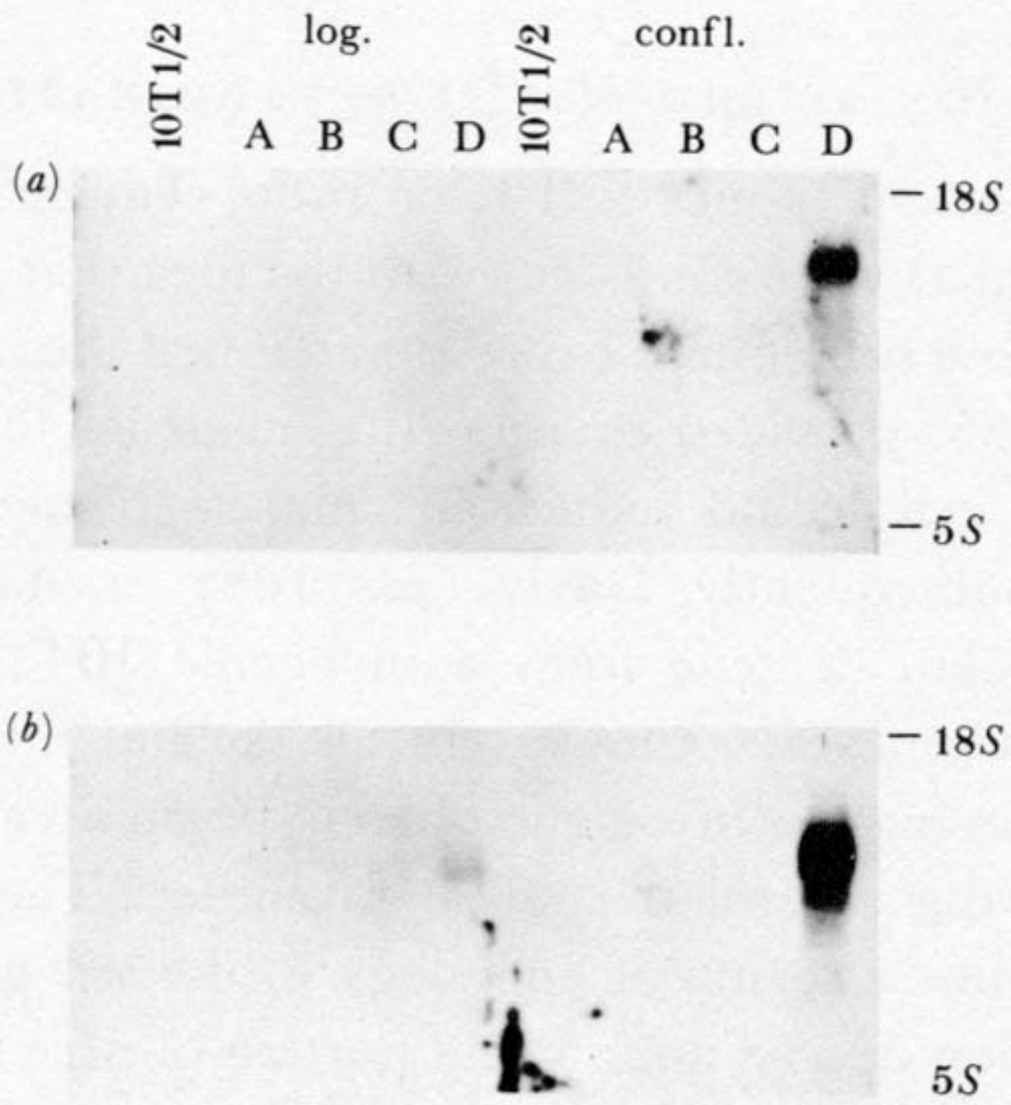

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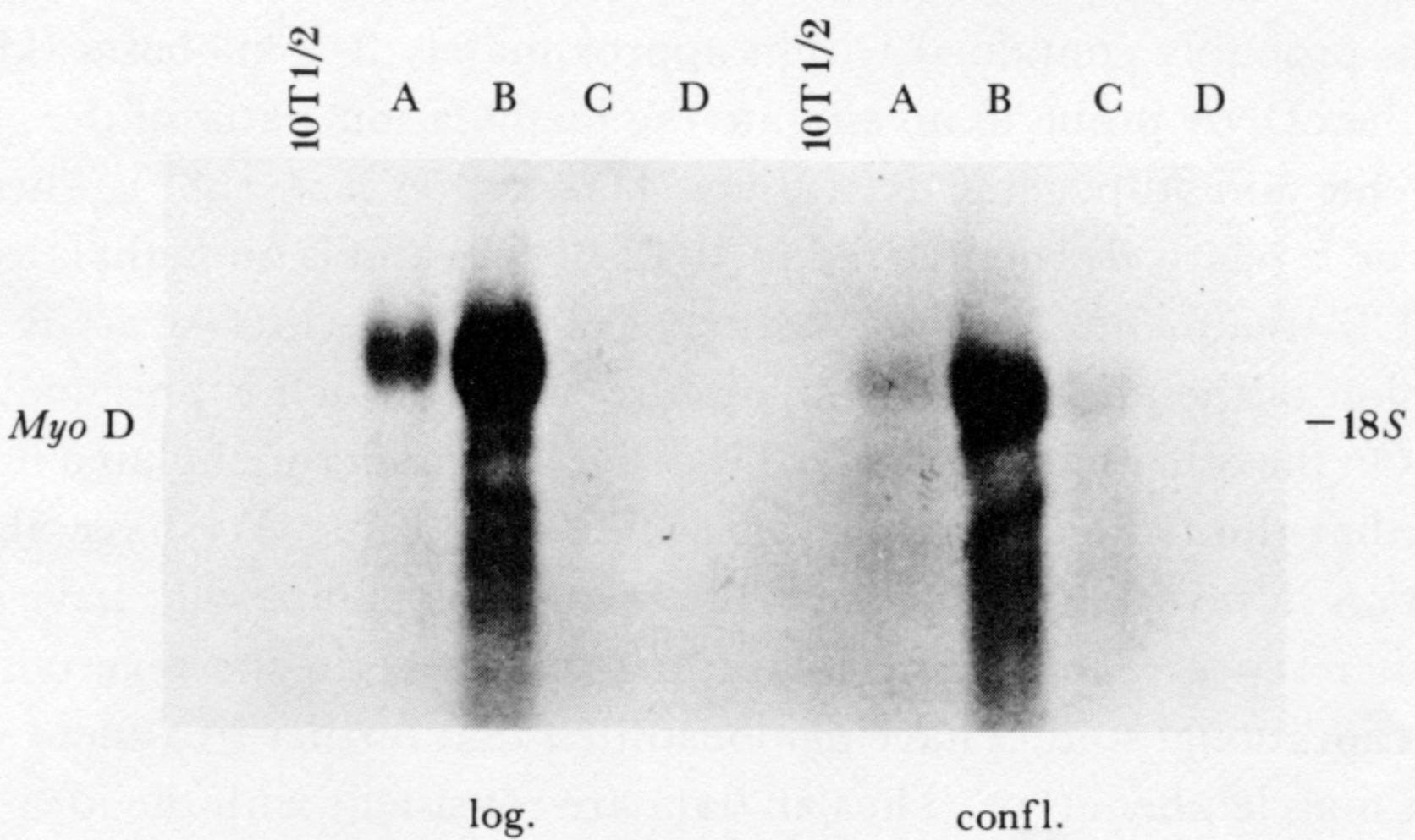

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