Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice

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ABSTRACT Translational control is a major form of regulating gene expression during gametogenesis and early development in many organisms. We sought to determine whether the translational repression of the protamine 1 (Prml) mRNA is necessary for normal spermatid differentiation in mice. To accomplish this we generated transgenic animals that carry a Prml transgene lacking its normal 3′ untranslated region. Premature translation of Prml mRNA caused precocious condensation of spermatid nuclear DNA, abnormal head morphogenesis, and incomplete processing of Prm2 protein. Premature accumulation of Prml within syncytiat spermatids in mice hemizygous for the transgene caused dominant male sterility, which in some cases was accompanied by a complete arrest in spermatid differentiation. These results demonstrate that correct temporal synthesis of Prml is necessary for the transition from nucleohistones to nucleoprotamines.

Haploid spermatid differentiation (spermiogenesis) in the mouse takes ~2 weeks and consists of 16 steps that are characterized by changes in nuclear morphology and acrosome development (1, 2). During spermiogenesis the histones on the DNA are gradually replaced by a class of proteins with intermediate basicity called the transition proteins (TPs) (3). The TPs are thought to participate in the removal of the histones and in the initial condensation of the spermatid nucleus (4, 5). Later in spermiogenesis the TPs are replaced with two small arginine-rich proteins called the protamines (6). The protamines clearly are not required for initiating sperm nuclear condensation because condensation begins before protamine synthesis, but they may have a role in completing it in later-stage spermatids and in maintaining it in mature spermatozoa.

A special feature of sperm morphogenesis is that global transcription from the haploid genome ceases several days before the completion of spermiogenesis and the synthesis of the transition proteins and the protamines (7, 8). To accommodate the cessation of transcription, the mRNAs that encode the TPs and the protamines are synthesized in round spermatids (steps 7–9), stored as cytoplasmic ribonucleoprotein particles for up to a week (steps 7–12), and finally translated in elongated spermatids (steps 12–16) (4, 9–11).

Transgenic studies have shown that the 3′ untranslated region (3′ UTR) of Prml mRNA is both necessary and sufficient to mediate translational repression during spermatid differentiation (12, 13). Recent transgenic analysis suggests that two different regions of the Prml 3′ UTR are important for translational repression and that each is sufficient for repression independent of the other (unpublished data). Proteins that bind to different regions of the Prml 3′ UTR RNA in vitro have been described and postulated to be involved in translational repression, although direct proof that they function as translational repressors in vivo has not yet been demonstrated (14, 15).

An important and unanswered question in sperm morphogenesis is whether the temporal expression of the testis nuclear basic proteins is actually necessary for normal spermatid differentiation. To address the importance of the translational repression of Prml mRNAs, we generated transgenic mice that fail to delay Prml mRNA translation. We show that premature translation of Prml mRNA causes precocious condensation of nuclear DNA and a failure to complete spermatid differentiation, resulting in sterility.

MATERIALS AND METHODS

Plasmid Construction and Transgenic Mice. The 4.5-kb transgene contains ~4.1 kb of Prml 5′-untranslated sequence extending 5′ of the transcriptional start site to a HindIII site at −4.1 kb, the 95-nt noncoding region of exon 1, the complete coding exons and intronic sequence of Prml (247 nt) (16), and the 105-nt 3′ noncoding region of the human growth hormone (hGH) gene (17). Previous studies have established that 4.1 kb of Prml 5′-flanking DNA is sufficient to direct spermatid-specific transcription upon a heterologous gene in transgenic mice (16). Transgenic mice were generated by pronuclear injection of fertilized eggs derived from (C57BL/6×SJL)F1 females mated with identical hybrid males as described (18).

RNA Analysis. Total RNA was isolated from dissected testes using the method of Chomczynski and Sacchi (19). RNA samples were separated on a 1.5% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose membrane, fixed by baking at 80°C, and hybridized at 45°C in 50% (vol/vol) formamide/5× standard saline citrate (SSC)/5× 50 mM NaPO4 (pH 6.5)/single-stranded DNA at 250 μg/ml/1× Denhardt’s solution/0.5% SDS/6.25% (wt/vol) dextran sulfate/α-32P-labeled DNA probes were prepared by random hexamer labeling. The blot was washed at a final stringency of 0.1× SSC/0.5% SDS at 50°C.

Basic Nuclear Protein Preparation. Crude nuclei were prepared by homogenizing testes in 20 mM Tris-HCl, pH 7.7/40 mM KCl/17 mM MgCl2/protease inhibitors ( aprostatin at 1 μg/ml, 1 mM benzamidine, leupeptin at 0.5 μg/ml, pepstatin A at 1 μg/ml, and 0.5 mM phenylmethylsulfonyl fluoride), filtering the homogenates through three layers of gauze, and centrifuging at 250 × g for 5 min at 4°C. The nuclei pellet was suspended in MKPT [20 mM Tris-HCl (pH 7.7)/40 mM KCl/110 mM MgCl2/protease inhibitors] supplemented with 1% Triton X-100 and 0.32 M sucrose and further purified

Abbreviations: Prml and Prm2, protamine 1 and 2, respectively; TPs, transition proteins; 3′ UTR, 3′ untranslated region; hGH, human growth hormone.

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by sedimenting through 1.1 M sucrose/MKPT as described (20). The nuclei pellet was resuspended in cold distilled water/protease inhibitors and sonicated for three 30-sec bursts, each at 60 W. Sonication-resistant spermatid nuclei were recovered by centrifugation at 6000 × g for 5 min at 4°C from the sonication-sensitive nuclei (supernatant). Basic nuclear proteins were isolated as described with some modifications (21). Briefly, the sonication nuclei were washed twice and dissolved in protamine extraction buffer [6 M guanidine-HCl/0.5 M Hepes (pH 7.5)/10 mM dithiothreitol] for 1 hr at room temperature. Cysteine residues were alkylated by adding vinylpyridine to a final concentration of 0.25 M and incubating the mixture for 1.5 hr at room temperature (22). HCl was added to a final concentration of 0.9 M and dialyzed overnight against 0.2 M HCl. Insoluble protein and DNA were recovered by centrifugation at 12,000 × g for 10 min. The proteins in the supernatant were precipitated with 20% trichloroacetic acid and washed with acidified acetone as described (23). Basic nuclear proteins from sonication-sensitive nuclei (the supernatant fraction) were prepared by extracting with 0.25 M HCl twice as described (23). Proteins in the pooled extract were precipitated with 20% trichloroacetic acid, resuspended in protamine extraction buffer, and processed as those from sonication-resistant nuclei. Basic proteins were dissolved in 8 M urea/0.9 M acetic acid/0.1 M 2-mercaptoethanol/1% methyl green and separated in polyacrylamide slab gels containing 15% acrylamide, 0.1% bisacrylamide, 6.2 M urea, 0.9 M acetic acid by a modification of a described method (24). The gels were prerun at 200 V for 2.5 hr in 0.9 M acetic acid. Protein samples were loaded and electrophoresed in fresh running buffer at 20 mA constant current for ~2 hr. The gels were stained in 0.2% naphthol blue-black/40% (vol/vol) ethanol/7% (vol/vol) acetic acid and destained in the same solution without dye.

**Immunoblot Analysis.** The proteins separated on an acrylamide gel were electrobotted onto an Immobilon-P filter (Millipore) in 0.7% acetic acid at 400 mA for 15 min. Half of the blot was stained in 0.25% Coomassie brilliant blue/45% methanol/10% (vol/vol) acetic acid and subsequently destained in 50% (vol/vol) methanol/10% acetic acid. The other half of the blot was blocked with 5% nonfat dry milk/phosphate-buffered saline (BPBS) and then incubated overnight at 4°C with the Hup2B (25) antibody diluted in BPBS. After washing one time for 20 min in BPBS/0.05% Tween 20 and twice in BPBS, the blot was sequentially incubated with biotinylated goat anti-mouse IgG antibody and horseradish peroxidase-conjugated streptavidin. Horseradish peroxidase was revealed with the chromogenic substrate 4-chloro-1-naphthol (Bio-Rad).

**RESULTS**

**Sterility of Transgenic Mice.** To prematurely express Prm1 protein in round spermatids, transgenic animals were generated that carry a chimeric gene containing the promoter, 5′ noncoding and coding regions of *Prm1*, and the hGH 3′ noncoding region as described in Materials and Methods. We expected that the transgene would be transcribed in round spermatids at the same time as the endogenous *Prm1* gene and that the transgenic mRNA would be translated prematurely in round spermatids because it lacked the *Prm1* 3′ UTR. The sterility of the transgenic founders and their male offspring was scored for histological phenotypes and expression levels. Among the different sterile founders and transgenic lines, histological analysis revealed two clearly distinguishable phenotypes. As detected by light microscopy, the majority of the sterile males contained late-stage spermatid defects charac-

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### Table 1. Summary of mouse lines that prematurely translate Prm1 mRNA

<table>
<thead>
<tr>
<th>Line</th>
<th>Expression level</th>
<th>Fertility</th>
<th>Transmission no./total no. offspring</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Founder</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11080</td>
<td>High</td>
<td>Sterile</td>
<td>NA</td>
<td>Early spermatid arrest</td>
</tr>
<tr>
<td>237</td>
<td>Moderate</td>
<td>Sterile</td>
<td>NA</td>
<td>Late spermatid defects</td>
</tr>
<tr>
<td>11085</td>
<td>Moderate</td>
<td>Sterile</td>
<td>NA</td>
<td>Late spermatid defects</td>
</tr>
<tr>
<td>11066</td>
<td>Moderate</td>
<td>Sterile</td>
<td>NA</td>
<td>Late spermatid defects</td>
</tr>
<tr>
<td>11060</td>
<td>Low</td>
<td>Fertile</td>
<td>0/30</td>
<td>Mosaic</td>
</tr>
<tr>
<td>11068</td>
<td>Moderate</td>
<td>Fertile</td>
<td>0/26</td>
<td>Mosaic</td>
</tr>
<tr>
<td>4</td>
<td>Moderate</td>
<td>Fertile</td>
<td>0/28</td>
<td>Mosaic</td>
</tr>
<tr>
<td>F1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11134</td>
<td>Low</td>
<td>Semifertile</td>
<td>45/124</td>
<td>ND</td>
</tr>
<tr>
<td>11135</td>
<td>High</td>
<td>Sterile</td>
<td>NA</td>
<td>Early spermatid arrest</td>
</tr>
<tr>
<td>11142</td>
<td>Moderate</td>
<td>Sterile</td>
<td>NA</td>
<td>Late spermatid defects</td>
</tr>
</tbody>
</table>

NA, not applicable; ND, not determined. Fertility of male founders and F1 male offspring of female founders was determined by cohabitation with multiple females for at least 1 mo. Semifertility in the 11134 line means that some F1 males are fertile and some are sterile. Expression level of the transgene refers to a qualitative assessment of transgenic mRNA levels as determined by Northern blotting (Fig. 3). Transmission refers to the fraction of F1 animals that inherit the transgene from a fertile transgenic male. Phenotype refers to the histological phenotype. In the early spermatid-arrest phenotype all seminiferous tubules show a uniform arrest at the round-spermatid stage (Fig. 1 C and F). The late-stage spermatid-defect phenotype refers to abnormal head morphogenesis in early elongating spermatids and the failure to release spermatids from the seminiferous epithelium (Fig. 1 B and E). Mosaic refers to a mixed histology, where either whole sections of seminiferous tubules or portions of tubules showing the late-stage spermatid-defect phenotype are present along with regions of normal histology (data not shown).
terized by abnormal head morphogenesis and a failure to complete the final stages of spermiogenesis (Fig. 1 B and E). A second phenotype, seen in founder animal 11080 and line 11135, was a complete developmental arrest at the round spermatid stage observed in every seminiferous tubule examined (Fig. 1 C and F). In some instances spermatids formed multinucleated cells, by relaxation of intercellular bridges and the convergence of cytoplasm (Fig. 1F). In both phenotypes no mature spermatozoa were detected in the epididymis (Fig. 1 H and I).

**Synthesis and Premature Translation of Prml mRNA.** On the basis of previous experiments (12), we expected that replacement of the 3' UTR of Prml with the 3' UTR of hGH would lead to premature translation of the transgenic Prml-hGH mRNA. We confirmed that Prml protein was synthesized early by using immunocytochemistry. Treatment of testis sections from males in lines 11142 and 11135 (Fig. 2A and B, respectively), with anti-Prml antibody showed that Prml was synthesized prematurely and that it was localized to the nucleus. We detected Prml in the testis from males in line 11142 in round and elongating spermatids (steps 7–12) and in line 11135 in round spermatids (steps 7–8), whereas Prml protein is normally first detected in step 12 (data not shown).

To determine whether the different histological phenotypes correlated with the level of transgene expression, Northern blot analysis was performed on RNA isolated from the testes of the transgenic mice (Fig. 3). Highest levels of transgene expression (founder 11080 and line 11135) correlated with the early spermatid-arrest phenotype, moderate levels of transgenic mRNA with late-stage spermatid-defect phenotype, and extremely low levels (line 11134) with semifertility. Theoretically, the spermatogenic phenotypes we observed could be due solely to increased amounts of Prml protein and not to its altered temporal expression. However, >40 lines of transgenic mice have been established that contain as much as three times the normal amount of Prml synthesized at the appropriate time during spermiogenesis (16, 28, 29), and in no case has this led to fertility defects. Thus, overexpression of Prml protein does not affect spermatid differentiation, but its time of synthesis does.

**Expression of TPs and Endogenous Protamines.** Given that premature expression of Prml protein occurs when the haploid genome is still engaged in the transcription of genes required for completion of spermiogenesis, one explanation for the observed phenotypes is that Prml protein disrupts the transcription of other postmeiotically expressed genes. To test this

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**Fig. 1.** Histological analysis of testes from sterile transgenic males. Low (A) and high (D) magnification of sections through control testis. The arrow points to the head of a normal elongating spermatid(es). Low (B) and high (E) magnifications of sections through testis of F1 male from 11142 line with the late-stage spermatid-defect phenotype. Tubules in B are highly disorganized; they contain numerous abnormal elongating spermatids and few late-stage elongated spermatids. The arrowheads in E point to a collection of elongating spermatids that have grossly abnormal heads. Low (C) and high (F) magnifications of testis sections from F1 male from 11135 line with early-stage spermatid-arrest phenotype. Note the absence of any elongating spermatids and the many uniformly staged round spermatids (rs). Seminiferous tubules in D, E, and F are all at the same developmental stage (XII) as characterized by the presence of spermatocytes in metaphase (m). G, H, and I show sections through the epididymis of a control, 11142, and 11135 male, respectively. Arrows point to the lumen of tubules that are; packed with sperm (G), completely devoid of sperm (H), or contain a few round spermatids, apparently sloughed from the seminiferous epithelium (I). Testes were fixed in Bouin’s solution, embedded in paraffin, sectioned, and stained with hematoxylin and periodic acid/Schiff reagent. Bar = 25 μm (A), 8 μm (D), and 50 μm (G).
A second hypothesis to explain the sterility phenotype is that premature synthesis of Prm1 affects the synthesis of the TPs and the endogenous protamines. To analyze synthesis of the various testis basic nuclear proteins, total testis basic proteins were isolated from sonication-sensitive and sonication-resistant nuclei and separated by acid-urea gel electrophoresis. Spermatid nuclei normally become sonication-resistant in wild-type mice after deposition of the TPs and protamines (Fig. 4A, lanes 1 and 2) (3). In animals with the late-stage spermatid-defect phenotype (Fig. 4A, lanes 6, 8, 10, and 16), we detected the presence of Prm1, transition protein 2 (TP2), and the precursor of Prm2, in sonication-resistant nuclei only. The absence of Prm1 in sonication-sensitive nuclei (Fig. 4A, lanes 5, 7, 9, and 15) suggests that accumulation of Prm1 leads to sonication resistance. In no animals did we observe levels of Prm1 protein in excess of that seen in normal control males, again supporting the conclusion that it is the time of Prm1 expression and not the absolute amount of Prm1 protein that is responsible for the spermiogenic phenotypes.

By protein analysis we cannot distinguish between Prm1 protein expressed from the transgene and from the endogenous Prm1 gene. However, translation of endogenous Prm1 mRNA is normally accompanied by significant shortening of its poly(A) tail, resulting in a heterogeneous population of transcripts ranging in size between \( \sim 400 \) and 560 nt (9). A shortened, and presumably deadenylated, Prm1 RNA is seen in the control nontransgenic samples and in RNA samples from all transgenic animals that show the late-stage spermatid-defect phenotype (Fig. 3, lanes 1, 3–11, and 13), suggesting that the endogenous Prm1 mRNA is translated in those animals.

Prm2 is normally synthesized as a precursor protein of 106 amino acids and is processed through a series of proteolytic cleavages to a final size of 63 amino acids (30, 31). Interestingly, animals that displayed the late-stage spermatid-defect phenotype only contained the largest of the unprocessed forms of Prm2 (Fig. 4A, lanes 6, 8, 10, and 16). To verify the identity of precursor, partially processed, and mature Prm2, we analyzed the basic proteins prepared from a normal testis, and from a founder and F1 male with the late-stage spermatid-defect phenotype, by immunoblot analysis. In the control

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**Fig. 3.** Northern blot analysis of RNA from founder and F1 transgenic males. Lanes 1–9 contain total testis RNA isolated from control or transgenic founders, and lanes 10–13 contain RNA isolated from control and F1 transgenic offspring. Each lane contains \( \sim 15 \mu \text{g} \) of total RNA isolated from the testes of sexually mature males. The blot was hybridized sequentially with \( ^{32} \text{P} \)-labeled DNA probes specific for each of the mRNAs. The Prm–hGH mRNA was detected with a probe prepared from a DNA fragment containing the hGH 3' UTR. The endogenous Prm1 mRNA was detected with a probe prepared from a DNA fragment containing the Prm1 3' UTR. The Prm2 and actin mRNAs were detected with probes prepared from their full-length cDNAs, respectively. A low level of Prm–hGH transcript was detected in founder 11060 (lane 6) upon longer exposures (data not shown).

Hypothesis Northern blot analysis was used to assess the mRNA levels from the endogenous Prm1 and Prm2 genes. Fig. 3 shows that, relative to actin RNA, the endogenous Prm1 and Prm2 transcripts are present in normal amounts in animals with both phenotypes, suggesting that a general block in global transcription is not responsible for the phenotypes observed.

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**Fig. 4.** (A) Analysis of total testis basic proteins from sonication-sensitive and sonication-resistant spermatid nuclei. Lanes 1–10 contain protein isolated from control and transgenic founders, and lanes 11–16 contain protein isolated from control and F1 transgenic offspring. Total basic testis proteins were prepared from sonication-sensitive (SS) and sonication-resistant (SR) nuclei, fractionated by acid-urea polyacrylamide gel electrophoresis, and detected by staining with 0.2% naphthol blue-black, as described. Positions of TP1; Prm1; and the precursor, partially processed, and mature forms of Prm2 are labeled. (B) Immunoblot analysis of precursor and processed forms of Prm2. Sonication-resistant testis nuclear basic proteins were prepared from a nontransgenic male, a transgenic founder animal with the late-stage spermatid defect (11066), and a transgenic F1 animal with the late-stage spermatid defect (11142-28). Proteins were fractionated by acid-urea/polyacrylamide gel electrophoresis, transferred to Immobilon-P filter (Millipore), and either stained with Coomassie blue (lanes 1–3) or incubated with an anti-Prm2 mouse monoclonal antibody (Hup2B) that was raised against human PRM2 (lanes 4–6). This antibody crossreacts with mouse Prm2 but does not crossreact with mouse Prm1. The primary antibody was detected with a biotinylated goat anti-mouse secondary antibody and streptavidin-conjugated horseradish peroxidase (Zymed and Vector Laboratories) with the chromogenic substrate 4-chloro-1-naphthol (Bio-Rad). Positions of Prm1; TP2; and the precursor, processed, and mature forms of Prm2, are indicated.
sample we detected several forms of Prm2 (Fig. 4B, lanes 1 and 4), whereas in animals 11066 and 11142-28 only the precursor and the largest of the partially processed forms were detected (Fig. 4B, lanes 2, 3, 5, and 6). Thus, mice with the late-stage spermatid-defect phenotype fail to make mature Prm2 protein, suggesting that transition from nucleohistones to protamines in these animals is incomplete.

**DISCUSSION**

Premature translation of Prm1 mRNA in transgenic mice resulted in a failure to produce mature sperm. In animals that expressed the highest levels of Prm1 protein, spermatogenesis was arrested uniformly at the round-spermatid stage. In animals that expressed lesser amounts of Prm1 protein, spermatogenesis continued beyond the round-spermatid stage but was highly abnormal. Both spermatogenic phenotypes are due to premature translation of Prm1 mRNA and not to its overexpression, as previous studies have clearly shown that overexpression of Prm1 protein at its normal time of synthesis does not adversely affect spermatogenesis (16, 28, 29). Furthermore, in none of the transgenic animals was the amount of Prm1 protein higher than the endogenous Prm1 protein level in control animals. Therefore, translational regulation of Prm1 mRNA is absolutely required for normal spermatid differentiation, and premature translation of Prm1 mRNA leads to dominant male sterility.

Spermatid nuclear condensation normally begins in the step-12 elongating spermatid and is thought to be mediated by the TPs (3). On the basis of the relative timing of events, it has been proposed that the TPs initiate nuclear condensation and that the protamines complete the process. Significantly, however, spermatids in animals with the early spermatid-arrest phenotype failed to make TPs but had become sonication-resistant, suggesting that Prm1 protein is responsible for the sonication resistance. In addition, we were unable to detect Prm1 protein in sonication-sensitive spermatid nuclei in animals with the early spermatid-arrest phenotype or the late-stage spermatid-defect phenotype. Together these experiments provide a direct demonstration that Prm1 protein mediates the precocious nuclear condensation that leads to sonication resistance; however, this process is probably abnormal.

In one transgenic founder male, and in all males in one transgenic line, premature expression of Prm1 protein resulted in a uniform arrest of spermiogenesis at the round-spermatid stage. Northern analysis suggests that the premature synthesis of Prm1 protein does not block global transcription; however, our data do not rule out the possibility that synthesis of specific mRNAs is affected by Prm1 synthesis and that it is the absence of these mRNAs that cause the developmental arrest. Uniformity of the developmental block suggests that if transcriptional defects are responsible for the arrest, genes that regulate spermiogenesis may be those affected.

In most transgenic animals, premature expression of Prm1 protein caused abnormal nuclear shaping, precocious nuclear DNA condensation, and a general failure to complete the latter stages of spermiogenesis. Again, Northern analysis suggests that global transcription is not affected and is, therefore, not responsible for the phenotype observed. Unlike the animals with the early spermatid-arrest phenotype, animals with the late-stage spermatid defects synthesize the TPs and the endogenous protamines. Significantly, however, processing of the Prm2 precursor protein is incomplete, and no mature Prm2 protein is produced, suggesting that the processing of Prm2 is coupled with its normal codeposition with Prm1 protein. The deposition of Prm1 protein on chromatin before synthesis of the TPs demonstrates that removal of the TPs is not required for Prm1 deposition; however, our data suggest that the deposition of Prm1 protein is abnormal and that it interferes with the normal events of spermiogenesis. In summary, these data suggest that the failure to complete spermatid differentiation is due to a failure to complete the transition from nucleohistones to nucleoprotamines and demonstrate that chromatin condensation depends on the correct temporal synthesis of the TPs and the protamines.

Spermatid nuclear shaping begins several days before synthesis of the TPs and protamines. Spermatid nuclear morphology is under genetic control (32), and the extranuclear forces that shape this shaping process are mediated, in part, by a microtubular array called the manchette (33). On the basis of the abnormal nuclear morphology in animals with the late-stage spermatid-defect phenotype, our data support the hypothesis that intranuclear forces, mediated by the process of chromatin condensation, may also contribute to the shape of the spermatid nucleus (34).

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**References**