Protein Masking of a Ribosomal RNA Epitope Is an Early Event in Afferent Deprivation-Induced Neuronal Death

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Cell death in the developing nervous system is regulated by both afferent synaptic activity and target-derived neurotrophic factors. Loss of afferent innervation via unilateral cochlea removal results in the death of 20–40% of neurons in the neonatal chick cochlear nucleus, nucleus magnocellularis (NM). The process of NM neuronal death involves structural and functional alterations in ribosomes, including decreased protein synthesis, loss of immunoreactivity for a monoclonal anti-ribosomal RNA (rRNA) antibody, Y10B, and eventual ribosome degradation. In the present report we confirm that the Y10B antibody binds specifically to ribosomes in chick NM neurons by electron microscopy. We then performed experiments designed to determine whether loss of rRNA immunoreactivity observed in NM neurons following cochlea removal involves induction of a protein–rRNA interaction. Brain stem tissue from animals subjected to unilateral cochlea removal was treated with protease prior to immunolabeling. Protease treatment restored rRNA immunoreactivity after 3 h of afferent deprivation, confirming that afferent deprivation induces protein–rRNA interactions which mask the Y10B epitope. Immunoprecipitation experiments confirmed that the Y10B antibody recognizes a specific rRNA sequence without posttranscriptional modification.

INTRODUCTION

The development of a functional neural circuit is regulated by both anterograde and retrograde influences (Oppenheimer, 1991). For example, competition for retrogradely transported trophic substances regulates the size of many developing neuronal populations (Thoenen et al., 1987; Oppenheimer, 1989; Hendry, 1992). When deprived of target-derived neurotrophic factors, either in vivo or in vitro, developing neurons undergo an active cell death process which resembles apoptosis (Martin et al., 1988; Oppenheimer et al., 1990; Scott and Davies, 1990; Edwards et al., 1991). The anterograde influence of afferent synaptic input on neuronal growth and survival has also been documented in a variety of systems (Guillery, 1973; Kalil, 1980; Born and Rubel, 1985; Clarke and Egloff, 1988; Catsicas et al., 1992; Galli-Resta et al., 1993; Yin et al., 1994; Akutagawa and Konishi, 1995; reviewed by Linden, 1994). Currently, it is not known whether the loss of afferent innervation elicits the same sequence of cellular events as those induced by deprivation of target-derived neurotrophic factors. Study of the intracellular changes induced by afferent deprivation may provide information about the mechanism of neuronal death following loss of anterograde influence.

Neuronal death following afferent deprivation has been well characterized in the auditory brain stem (reviews: Rubel and Parks, 1988; Rubel et al., 1990). Neuronal population size in the primary brain stem auditory nucleus of mammals, the anteroventral cochlear nucleus (AVCN), and its avian homologue, nucleus magnocellularis (NM), is regulated by the anterograde influence of eighth nerve synaptic input (Trune, 1982; Born and Rubel, 1985, 1988; Hashisaki and Rubel, 1989). In immature animals, 20–40% of AVCN or NM neurons will die fol-
lowing loss of synaptic input through surgical removal of the ipsilateral cochlea or blockade of synaptic activity from the eighth nerve with the sodium channel blocker tetrodotoxin (TTX) (Born and Rubel, 1985, 1988; Hashi-
saki and Rubel, 1989).

Several metabolic and structural alterations precede cell death in afferent-deprived auditory neurons. Within 1 h, cessation of afferent synaptic activity produces a rapid but reversible decrease in protein synthesis by NM or AVCN neurons (Steward and Rubel, 1985; Born and Ru-
bel, 1988; Sie and Rubel, 1992). By 6 h after the loss of afferent input, NM neurons segregate into two populations. One group of neurons incorporates $^3$H-amino acid at a rate slightly decreased from intact NM neurons, while the remainder appear to have entirely ceased protein syn-
thesis (Steward and Rubel, 1985; Born and Rubel, 1988). This latter group also shows complete dissociation of polyribosomes, loss of ribosomes from endoplasmic reticulum, lack of a normal complement of monosomes, and the appearance of ribosomal debris (Rubel et al., 1991).

These two populations of NM neurons appear to con-
tinue down separate pathways unless eighth nerve activity is restored within the next 6–12 h (Born and Rubel, 1988; Garden et al., 1994). Most, if not all, of the neurons that have ceased protein synthesis and show a loss of ribosomal integrity undergo rapid cell death by 2–3 days after the beginning of afferent deprivation. The re-
main ing neurons appear to show partial restoration in the level of protein synthesis and other metabolic proper-
ties (Rubel et al., 1990) and survive for at least 2 months if not indefinitely. The survival of the majority of NM neurons appears dependent on mitochondrial biogenesis occurring during the first 24 h of afferent deprivation.

Further investigations of ribosomal changes resulting from afferent deprivation have employed a monoclonal anti-ribosomal RNA (rRNA) antibody, Y10B. Y10B has been shown to immunoprecipitate whole mammalian rib-
osomes as well as rRNA stripped of associated proteins (Lerner et al., 1981). Experiments reported here also demon-
strate that the Y10B antibody specifically immuno-
stains ribosomes in chick NM neurons. It has been previ-
ously demonstrated that afferent deprivation induces a biphasic change in the pattern of rRNA immunoreac-
tivity in NM neurons. One to 3 h following cessation of synaptic activity (several hours before evidence of ribo-
some degradation appears), nearly all afferent-deprived NM neurons demonstrate decreased rRNA immunoreac-
tivity, suggesting that a modification of the Y10B epitope has occurred. After longer term deprivations, two popu-
lations of NM neurons appear, those neurons that regain Y10B immunoreactivity, and those remaining unlabeled by this antibody (Garden et al., 1994). Immunohistochem-
istry with the Y10B antibody on tissue labeled for amino acid incorporation demonstrates that NM neurons not synthesizing protein 6 h after cochlea removal also fail to regain rRNA immunoreactivity (Garden et al., 1994). Those NM neurons failing to synthesize protein also show evidence of ribosome destruction by electron micro-
scopy (EM) (Rubel et al., 1991), demonstrating a corre-
lation between sustained loss of Y10B immunoreactivity and eventual loss of ribosome integrity.

The early reduction in Y10B labeling was initially puzzling because in the first 3 h after cochlea removal there is very little ultrastructural evidence of ribosome degra-
dation (Rubel et al., 1991). In the absence of ribosome degradation, one possible explanation for this loss of rRNA immunoreactivity is a reversible structural change in ribosomes. Possible mechanisms include: (1) modifi-
cation of a protein, such that it binds and masks the Y10B epitope; (2) alteration in the conformation of rRNA (e.g., change in folding); (3) enzymatic degradation of rRNA resulting from the action of an induced RNase (e.g., RNase H); and (4) changes in posttranscriptional modification of rRNA (e.g., methylation) if the Y10B epitope includes a site on the ribosome normally containing modified rRNA.

To determine whether the loss of Y10B immunoreac-
tivity following afferent deprivation involves a confor-
mational change in the ribosome, two approaches were taken. First, following unilateral cochlea removal, brain-
stem tissue was treated with proteinase K to determine if a protein had been induced to mask the Y10B epitope. The results of this manipulation suggest that protein masking is involved in the early loss of rRNA immunore-
activity, but does not explain the loss of Y10B label after 9 h of afferent deprivation. Second, we further character-
ized the nature of the Y10B epitope. Our results indicate that the Y10B epitope consists of a specific base sequence; biosynthesis of this epitope does not require posttranscrip-
tional modification.

RESULTS

The Y10B Antibody Specifically Recognizes Chick Ribosomes

The Y10B antibody is a monoclonal anti-rRNA anti-
body obtained from a panel of monoclonal anti-nucleic acid antibodies generated using a genetic mouse model of autoimmune disease (Lerner et al., 1981). Previous ex-
periments demonstrated that the Y10B antibody recog-
nizes a cytoplasmic epitope in chick neurons (Garden et al., 1994). To determine whether the Y10B antibody specifically recognizes ribosomes in cells of the chicken nervous system, brainstem sections were labeled with Y10B and immunogold for EM immunohistochemistry. The Y10B antibody specifically labels free ribosomes, polysomes, and endoplasmic reticulum-associated ribosomes at the EM level (Fig. 1). The antibody also recognizes rRNA in the nucleolus (data not shown) but fails to label other cytoplasmic components of the neuron. Eighth nerve fiber synaptic endings shown in Fig. 1A do not contain ribosomes and are not labeled by the Y10B antibody. Controls, without primary antibody exhibited a nonspecific background pattern of immunogold label.

These findings demonstrate that the Y10B antibody binds to ribosomes and newly synthesized nucleolar rRNA in NM neurons.

Protein Binding Blocks rRNA Immunoreactivity after 3 h of Afferent Deprivation

To determine if afferent deprivation induces a protein–rRNA interaction masking the Y10B epitope, adjacent sections were treated with or without protease prior to immunolabeling. Three hours after unilateral cochlea removal, afferent-deprived NM neurons in untreated tissue demonstrate a significant decrease in Y10B immunoreactivity compared to control neurons in the NM contra-
FIG. 2. Tissue from an animal sacrificed 3 h after cochlea removal and stained with the Y10B antibody to rRNA is shown. Neurons of the primary auditory brain stem of neonatal chicks from the side of the brain contralateral to cochlea removal are shown in (A). Activity-deprived neurons from the NM ipsilateral to cochlea removal are displayed in (B). Note the uniform decrease in Y10B label observed in NM neurons which underwent 3 h of activity deprivation. The contralateral (C) and ipsilateral (D) NM from the tissue section adjacent to the one pictured in A and B were treated with Proteinase K prior to Y10B immunohistochemistry. Note the similarity in the amount of Y10B label between the intact (C) and afferent-deprived (D) NM. Scale bar, 10 μm.

lateral to cochlea removal (Figs. 2A and 2B). In the adjacent protease-treated section (Proteinase K, 20 μg/ml, for 7.5 min prior to Y10B immunohistochemistry), afferent-deprived NM neurons do not exhibit a decrease in the amount of rRNA immunoreactivity (Figs. 2C and 2D). This finding supports the hypothesis that the initial loss of Y10B immunoreactivity in afferent-deprived NM neurons results from protein masking of the Y10B epitope.

One alternative explanation for the effect of protease treatment on Y10B immunoreactivity is that removing protein increases the ability of antibodies to penetrate the tissue section. Increased antibody penetration might result in saturation of the peroxidase and artifactual normalization of immunohistochemical reaction product density between afferent-deprived and intact NM neurons. To control for the possibility of a saturation artifact, the experiment was repeated with a 10-fold dilution in the concentration of primary antibody. The lower concentration of Y10B antibody reduces the density of immunohistochemical reaction product to a very low level,
but does not alter the impact of protease treatment on Y10B labeling of afferent-deprived NM neurons (data not shown). This finding demonstrates that the effect of protease treatment is not an artifact of peroxidase saturation. Additionally, protease treatment makes nucleolar rRNA available for Y10B binding (small arrows in Fig. 2C). Despite the high concentration of rRNA in the nucleolus and the ability of the Y10B antibody to recognize nucleolar rRNA in ultra-thin sections prepared for transmission electron microscopy, the nucleolus in 10-μm sections demonstrates minimal above-background Y10B label prior to protease treatment.

The optical density (OD) of the immunohistochemical reaction product in individual NM neurons was quantified using microdensitometry techniques described previously (Garden et al., 1994). Because the average density of immunohistochemical reaction product varies between sections, raw OD scores could not be used to compare different sections. Raw OD scores are therefore normalized to z-scores for every section analyzed (see experimental procedures). Using the z-score normalization procedure, every neuron is given a score based on the number of standard deviations its OD is displaced from the average OD obtained from control NM neurons (receiving normal afferent input) on the contralateral side of the same tissue section. Therefore, a neuron with a z-score of 0 has an OD equal to the mean OD for normally active NM neurons in the same anatomic plane. A neuron with a z-score of -2 has an OD of Y10B immunolabel 2 standard deviations less than the mean OD for control NM neurons from the opposite side of the brainstem.

Distribution plots showing the normalized staining density of all afferent-deprived or control NM neurons from four animals are displayed in Fig. 3. The upper panel shows the distribution plot obtained from tissue subjected to the conventional immunohistochemical procedure. Afferent-deprived neurons are represented by light bars and control neurons by black bars. Three hours after cochlea removal, the distribution of z-scores in afferent-deprived NM neurons is shifted to more negative z-scores (mean z-score -2.38, from previously reported data in Garden et al., 1994), demonstrating the decrease in Y10B immunoreactivity for this period of afferent-deprivation. The lower panel shows the distribution plot obtained from the same four animals when adjacent tissue sections were treated with Proteinase K prior to Y10B immunolabeling. The distribution of afferent-deprived z-scores now overlies the distribution of z-scores from normally innervated NM neurons. Two-way ANOVA revealed a significant interaction (P < 0.001) between the effects of protease treatment and afferent deprivation on the raw OD scores and of z-scores 3 h after cochlea removal, demonstrating that the effect of afferent deprivation on Y10B immunoreactivity is significantly restored by treating tissue with protease. In each animal the OD scores between deprived and nondeprived NM neurons were significantly different before protease treatment (P's < 0.01) but this difference was eliminated by protease treatment.

These results demonstrate that loss of rRNA immunoreactivity after 3 h of afferent deprivation does not result from destruction of the Y10B epitope. After protease treatment, the Y10B epitope is available for binding. These findings also suggest that alteration of rRNA immunoreactivity at this stage of afferent deprivation is due to masking of the Y10B epitope by a cytoplasmic protein in NM neurons.
Protein Binding Does Not Impact rRNA Immunoreactivity after 9 h of Afferent Deprivation

After longer periods of afferent deprivation, a portion of afferent-deprived NM neurons demonstrates complete loss of protein synthesis and ultrastructural evidence of ribosome destruction (Born and Rubel, 1985, 1988; Steward and Rubel, 1985; Rubel et al., 1991). Between 6 and 12 h after cochlea removal, rRNA immunoreactivity returns in some NM neurons, but those cells which have lost the capacity to synthesize protein remain unlabeled (Garden et al., 1994). To determine whether protein masking of the Y10B epitope accounted for the changes in Y10B immunoreactivity 9 h after cochlea removal, adjacent sections were labeled with Y10B before (Figs. 4C and 4D) and after (Figs. 4A and 4B) protease treatment. Neurons unlabeled by the Y10B antibody are present in the activity-deprived NM of both sections in approximately equal numbers (large arrows in Figs. 4B and 4D). Protease treatment does not appear to impact the number
FIG. 5. Grouped (n = 5) OD distribution plots demonstrate the lack of change in the distribution of immunolabel for the Y10B antibody in NM neurons subjected to 9 h of afferent deprivation. (A) Before protease treatment the distribution of OD z-scores for afferent-deprived NM neurons is skewed or bimodal with a subset of cells obtaining scores in the range for normally afferented NM neurons and the remaining cells obtaining scores which place them outside the normal distribution. (B) After protease treatment, the distribution of OD z-scores remains very similar in shape to the distribution obtained prior to protease treatment.

of neurons demonstrating loss of Y10B immunoreactivity 9 h after cochlea removal, but its effect on this tissue section is evidenced by Y10B label of the nucleolus (small arrows in Fig. 4C). This finding indicates that 9 h after loss of synaptic input, removing tissue proteins cannot expose an intact Y10B epitope in the subpopulation of afferent-deprived NM neurons demonstrating reduced rRNA immunoreactivity.

OD z-score distribution plots from animals sacrificed after 9 h of activity deprivation (n = 5) do not demonstrate a significant effect of protease treatment on the pattern of Y10B immunoreactivity (Fig. 5). There appears to be a small shift toward an OD distribution that is closer to the OD distribution of neurons from the contralateral NM. This small shift may be due to a lingering impact of the protein masking that was prominent at earlier times following loss of afferent input. Two-way ANOVA did not yield a significant interaction (P > 0.05) between protease treatment and cochlea removal on the optical density of Y10B immunohistochemical reaction product after 9 h of afferent deprivation.

To quantify the size of the subpopulation of afferent-deprived NM neurons demonstrating a significant decrease in the amount of Y10B label, any NM neuron with a z-score of less than -2 (2 standard deviations below the mean OD for control NM neurons) was considered Y10B-negative. The portion of NM neurons designated as Y10B-negative 3, 6, or 9 h following cochlea removal from adjacent tissue sections before and after protease treatment is displayed in Fig. 6. Three hours after loss of afferent input, 62% of affected NM neurons are Y10B-negative before protease treatment. After protease treatment, only 3% of Y10B-negative neurons remain. By 6 h following cochlea removal, protease treatment still reduces the percentage of Y10B-negative neurons from 41 to 24%, but many neurons remain unlabeled by the Y10B antibody. This indicates that after 6 h of afferent deprivation, a portion of NM neurons demonstrates a loss of

FIG. 6. Protease treatment significantly decreases the percentage of Y10B negative NM neurons which appear 3 h after cochlea removal. The effect of protease decreases with increasing time after cochlea removal. The gray bars represent the mean percentage of afferent-deprived NM neurons which were Y10B negative without protease treatment. The black bars represent the mean percentage of Y10B negative neurons in the afferent-deprived NM after the tissue was treated with protease. Asterisk indicates a significant decrease in the mean percentage Y10B negative neurons (P < 0.01).
FIG. 7. A 1% agarose gel containing the immunoprecipitation products from three species stained with ethidium bromide and visualized by uv transillumination. Lanes 1–3 contain RNA from chick brain, lanes 4–6, RNA from Xenopus oocytes, and lanes 7–9, the 1S and 25S rRNAs from Saccharomyces cerevisiae. +, positive control, 2 μg RNA before immunoprecipitation; IP, immunoprecipitation product with the Y10B antibody; −, negative control, immunoprecipitation without the Y10B antibody. The Y10B antibody recognizes the 1S and 2S rRNAs from all three species. None of the negative control lanes contained any immunoprecipitation products.

Y10B reactivity that cannot be unmasked by protease treatment, suggesting that these neurons have sustained a second ribosome modification in addition to blocked accessibility of the Y10B epitope. Nine hours after cochlea removal, there is no impact of protease treatment on the size of the Y10B-negative subpopulation of NM neurons. This finding indicates that after 9 h ofafferent deprivation, removal of tissue proteins fails to expose the Y10B epitope to the antibody in those neurons showing dramatically reduced immunoreactivity. Two-factor ANOVA demonstrated a significant effect of protease treatment (P < 0.005) and a significant interaction between treatment and time (P < 0.02). Post hoc comparisons using Fisher's protected LSD test showed a significant (P < 0.01) decrease in the number of Y10B-negative neurons 3 h after cochlea removal. However, at the 6- or 9-h time points, the percentage of NM neurons remaining Y10B-negative following protease treatment is not significantly different from untreated tissue (P > 0.10), and is roughly equivalent to the percentage of NM neurons with ultrastructural evidence of ribosome destruction 6 h after cochlea removal (Rubel et al., 1991).

The Y10B Antibody Immunoprecipitates Several rRNAs

Characterization of the Y10B epitope may aid in identifying cellular mechanisms involved in the modification and/or destruction of ribosomes observed in afferent-deprived NM neurons. The Y10B antibody immunoprecipitates whole ribosomes as well as all sizes of rRNA (28S, 18S, 5.8S, and 5S) from protein-extracted nucleic acid preparations (Lerner et al., 1981). It is not currently known whether the Y10B epitope results from a posttranscriptional modification of rRNA or a specific structural motif repeated in different rRNAs.

As a means of characterizing the Y10B epitope we sought to develop a procedure to immunoprecipitate nucleic acids that bind to this antibody. We employed the procedure outlined in Steitz (1989) to determine what types of rRNAs the Y10B antibody would bind. The Y10B antibody will immunoprecipitate large rRNAs (18S and 28S or 25S in yeast) from three different species: chick, Xenopus leavis, and Saccharomyces cerevisiae (Fig. 7). Control immunoprecipitations with no Y10B added to the protein A–Sepharose demonstrate the specificity of the immunoprecipitation procedure. The Y10B antibody was previously shown to recognize smaller (5S and 5.8S) rRNAs from mammalian cells (Lerner et al., 1981). To confirm that the Y10B antibody binds smaller chick or X. leavis rRNAs, immunoprecipitation products of these species were run on polyacrylamide gels (data not shown). The antibody recognizes chick 5.8S rRNA and two 5S RNAs characteristic of chick tissue (Lazar et al., 1983). The 5.8S rRNA from X. leavis is also recognized

FIG. 8. Diagram of the 5.8S rRNA clone and the restriction fragments generated to localize the region of the Y10B epitope.
TABLE 1
The Binding Characteristics of the in Vitro-Transcribed Sense and Antisense Xenopus leavis 5.8S rRNA in the Y10B Immunoprecipitation Assay

<table>
<thead>
<tr>
<th>Probe</th>
<th>Y10B binding</th>
<th>PAS binding</th>
<th>IgG2a binding</th>
<th>Cold 5.8S competition</th>
<th>Total RNA competition</th>
<th>tRNA competition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8S rRNA sense strand</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
<td>Yes (75%)</td>
<td>Yes (91%)</td>
<td>None</td>
</tr>
<tr>
<td>5.8S rRNA antisense strand</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
<td>Yes (40%)</td>
<td>Yes (90%)</td>
<td>Not done</td>
</tr>
</tbody>
</table>

*Note.* Both sequences bound the Y10B antibody. Neither sequence was bound by the protein A or purified IgGα. An excess of cold in vitro-transcribed 5.8S rRNA or total RNA could compete off both radiolabeled 5.8S rRNA probes, but an excess of tRNA could not compete with the 5.8S rRNA for Y10B binding.

by the Y10B antibody (see also Fig. 8), but 5S rRNA in this species appears to have less affinity for the antibody. The Y10B antibody does not immunoprecipitate tRNAs or other small RNAs. These results demonstrate that the Y10B antibody can bind to tRNA which has been separated from ribosomal proteins. These findings also suggest that the Y10B epitope is a highly conserved motif in rRNAs from divergent species.

The Y10B Antibody Recognizes in Vitro-Transcribed rRNA

The ability of the Y10B antibody to recognize many different types of rRNA suggests that the epitope might involve a posttranscriptional modification of rRNA as opposed to a widely conserved specific sequence. In vitro-transcribed rRNA was employed to determine whether the Y10B antibody recognizes sequence alone or if posttranscriptional modification is required to create the Y10B epitope. Both sense and antisense strands of the X. leavis 5.8S rRNA were transcribed in vitro with $^{35}$SUTP to generate a radiolabeled probe for immunoprecipitation binding studies. The results of several binding studies are presented in Table 1. The Y10B antibody recognizes the sense strand of in vitro-transcribed 5.8S rRNA, but it also recognizes the antisense strand of the 5.8S rRNA.

While these results demonstrate that the Y10B antibody is capable of binding RNA which has not undergone any posttranscriptional modification, they also suggest that our immunoprecipitation procedure may allow nonspecific binding when only a single RNA is available for binding. Several measures were taken to assess the possible reasons for apparent nonspecific binding to both sense and antisense strands of the 5.8S rRNA. To assure that these results are not secondary to covalent transfer of $^{35}$S to the antibody or protein A, the experiments were repeated with $^{32}$PUTP and the same results obtained. Control immunoprecipitations either without any antibody or with purified IgG2a instead of Y10B produced no above background radioactivity in the immunoprecipitation products. Additionally, nonradioactive in vitro-transcribed 5.8S RNA or total RNA compete successfully with all three radio-labeled in vitro-transcribed RNAs while tRNA (which did not demonstrate immunoreactivity in the original nonradioactive immunoprecipitation) does not compete for Y10B binding. The results of these control experiments suggest that binding of both sense and antisense rRNA in the immunoprecipitation assay resulted from the ability of the Y10B antibody to recognize both RNA molecules as opposed to a problem with the assay procedures.

The Y10B Antibody Recognizes Specific rRNA Sequence

There are several possible explanations for the finding that Y10B antibody binds to both sense and antisense strands of the 5.8S rRNA. One possible explanation is that the antibody recognizes the uncapped 5' end of any RNA and its in vivo specificity is determined by capping of mRNAs transcribed in vivo. This possibility was eliminated by synthesizing capped 5.8S rRNA transcripts and demonstrating that the Y10B antibody was capable of binding the capped transcripts (data not shown). A second possible explanation for the ability of the Y10B antibody to bind these three transcripts might be that the Y10B epitope is so small and nonspecific (e.g., an individual base or the ribose backbone) that Y10B has significant affinity for any RNA in isolation. Alternatively, the Y10B epitope could involve secondary RNA structure often coded for by complementary sequences and therefore may be maintained in the antisense 5.8S transcript.
To distinguish between the second and third explanations, radiolabeled 5.8S rRNA transcripts of three different sizes were generated to determine if the Y10B epitope could be localized more specifically within the 5.8S rRNA. Figure 8A is a schematic of the plasmid with the 5.8S rRNA gene inserted in between the two promoter sites. By employing different restriction enzymes to linearize the plasmid, three different fragments still under the control of the T7 promoter (Figs. 8B and 8C) were generated.

The results of two immunoprecipitation experiments comparing the binding behavior of the three fragments are displayed in Table 2. The smallest 51-base fragment is less effectively bound to the Y10B antibody compared to the 118-base fragment and the full-length 5.8S rRNA. Electrophoretic separation of radiolabeled 5.8S rRNA, the two smaller 5.8S rRNA fragments and their corresponding immunoprecipitation products are shown in Fig. 9. Immunoprecipitation of the 5' 51-base fragment (lane 3) demonstrates that Y10B does not recognize the 51-base fragment but will recognize the higher molecular weight fragments generated during in vitro transcription. The higher molecular weight bands likely result from incomplete digestion of the plasmid due to several additional Alul sites downstream from the site intended for generating the 51-base fragment. The 118-base fragment appears as two bands of similar size (lanes 4 and 6) in both the total transcript and the immunoprecipitation lane. The larger of the two bands also results from incomplete HaeIII digestion due to a second HaeIII site 18 bases downstream from the intended HaeIII site. The 118-base fragment contains the information needed for Y10B binding, but the 51-base fragment does not. Therefore, the Y10B epitope must require the information contained within the 68 bases in the 118-base fragment which are not present in the 51-base fragment. This finding supports the hypothesis that the Y10B epitope is a specific RNA sequence or perhaps the secondary and tertiary structure which that sequence confers.

### DISCUSSION

The molecular mechanisms involved in neuronal death following the loss of afferent input have not been studied as thoroughly as those associated with neuronal death following trophic factor deprivation (Johnson and Deckwerth, 1993). However, previous results demonstrating rapid structural and functional changes in ribosomes following loss of afferent input contribute to the belief that studying ribosomes with the Y10B antibody will shed light on the process of neuronal death in afferent-deprived NM neurons. This supposition is bolstered by the electron microscopic evidence presented above, showing immunolocalization of this antibody to ribosomes in the cytoplasm of NM neurons. The experiments described here were designed to characterize the ribosome modification responsible for early loss of Y10B im-

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**TABLE 2**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Trial No. 1 (%)</th>
<th>Trial No. 2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alul fragment (3' 51 bases)</td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>HaeIII fragment (3' 118 bases)</td>
<td>95</td>
<td>70</td>
</tr>
<tr>
<td>Sense strand (162 bases)</td>
<td>82</td>
<td>53</td>
</tr>
<tr>
<td>Antisense strand (162 bases)</td>
<td>67</td>
<td>62</td>
</tr>
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**FIG. 9.** Autoradiogram of a 10% acrylamide gel containing the immunoprecipitation products of the in vitro-transcribed 5.8S rRNA and the two smaller restriction fragments. Lanes 1–3 contained the 51-base *Alul* fragment, lanes 4–6, the 118-base *HaeIII* fragment, and lanes 7–9, the whole 5.8S rRNA. +, positive control, in vitro-transcription product before immunoprecipitation; IP, immunoprecipitation product with the Y10B antibody; −, negative control, immunoprecipitation without the Y10B antibody. The major band in lane 1, the 51-base *Alul* fragment in the positive control lane is not seen in the Y10B immunoprecipitation lane (lane 3) where as the larger fragments in this preparation are recognized by the Y10B antibody. The *HaeIII* fragments are also recognized by the Y10B antibody (lane 6). None of the negative control lanes contain any radiolabeled RNA.
munoreactivity in afferent-deprived NM neurons. Four possible mechanisms for reducing Y10B immunoreactivity include: protein blockade of the Y10B epitope; conformational alteration in rRNA structure; enzymatic degradation of rRNA; and alteration in a posttranscriptional rRNA modification required for Y10B binding. Removing proteins from tissue of animals sacrificed after 3 h of afferent deprivation returned Y10B immunoreactivity to control levels. This finding suggests that activity deprivation induces an association between a protein and the Y10B epitope, thus preventing Y10B binding.

The ability of proteolysis to restore Y10B immunoreactivity after short term activity deprivation is inconsistent with several of the possible mechanisms noted above. If the initial loss of Y10B immunoreactivity involves an alteration in the conformation of rRNA, removal of proteins from fixed tissue would not be expected to restore immunoreactivity. Similarly, the effect of an induced RNase would not be reversed in protease-treated tissue. However, if the Y10B antibody recognizes a posttranscriptional modification that is altered after loss of synaptic activity, protease treatment could increase Y10B immunoreactivity by removing ribosomal proteins, thereby opening more modified bases for antibody binding. Immunoprecipitation experiments described here demonstrate that the Y10B antibody recognizes a specific base sequence and is capable of recognizing in vitro-transcribed rRNA without posttranscriptional modification. These findings demonstrate that posttranscriptional modification of rRNA is not required for Y10B binding. Thus, it is unlikely that reversible base alterations are responsible for changes in Y10B immunoreactivity observed in afferent-deprived NM neurons.

The remainder of this discussion will consider four issues. First, RNA-protein interactions and their possible role in cellular regulation will be addressed. Second, an in-depth discussion of what can be inferred about the Y10B epitope from the binding studies described here will be presented. Third, how the findings associated with neuronal death after deafferentation fit into the general scheme of programmed cell death, or apoptosis, will be discussed. Finally, the question of how these findings fit into the cascade of events leading to deafferentation-induced neuronal death will be addressed.

**RNA–Protein Interactions**

The finding that removal of proteins from NM neurons results in the return of Y10B immunoreactivity after 3 h of afferent deprivation suggests that an early ribosome modification in the cell death process involves a reversible protein–rRNA interaction. The importance of RNA–protein interactions in the regulation of cellular functions is not unprecedented. Some proteins can be covalently linked to RNA, as in the binding of the p53 tumor suppressor gene product to 5S rRNA (Fontoura et al., 1992). Another example of protein–RNA binding appears to have a regulatory role in translation. The human immunodeficiency virus (HIV-1) encodes a protein, TAT, that promotes transcription and translation of viral proteins through its ability to interact with an RNA element, TAR, found in the 5' end of viral mRNAs. Two additional proteins from *Xenopus* oocytes have been shown to interact with TAR (Braddock et al., 1993). The existence of eukaryotic proteins capable of interacting with a viral protein–RNA translational regulation scheme suggests that similar protein–RNA interactions may be involved in a conserved mechanism for translational regulation in eukaryotic cells.

The protein–RNA interaction that produces a reduction in Y10B immunoreactivity in afferent-deprived NM neurons may be involved in the loss of translational activity and/or the eventual ribosome degradation that precedes cell death in this population. Earlier studies have shown that translation is down-regulated in afferent-deprived NM neurons (Steward and Rubel, 1985; Born and Rubel, 1988) and the possibility that loss of Y10B immunoreactivity reflects an event regulating translation is an intriguing hypothesis. If this were the case, the protein responsible for masking of the Y10B epitope might also be involved in the regulation of translation. Identification and purification of the Y10B epitope binding protein might thus prove valuable to studies of ribosome regulation in neuronal death.

**The Nature of the Y10B Epitope**

Identification of the ribosome structural modification which accounts for the loss of Y10B immunoreactivity in afferent-deprived NM neurons necessitates an understanding of the curious pattern of specificity of the Y10B antibody. Using electron microscopy we have shown that the Y10B antibody binds specifically to ribosomes in the neuronal cytoplasm. Further analyses have revealed that the Y10B antibody specifically recognizes several different rRNAs from diverse species, yet there is no known sequence element which is common to all of these RNAs. This suggests that the Y10B epitope may represent some common element of rRNA secondary structure, perhaps in combination with a limited degree of sequence specificity. The ability of the Y10B antibody to bind in vitro-
transcribed rRNA confirmed that posttranscriptional modifications are not needed to confer Y10B immunoreactivity. The selective binding of only the larger fragments of *in vitro*-transcribed 5.8S rRNA by the Y10B antibody narrowed down the region containing the Y10B epitope to 68 bases in the middle of the 5.8S molecule.

The findings presented on Y10B binding suggest several criteria for the structure of the rRNA epitope which this antibody recognizes. First, the Y10B epitope requires the structural information found in the 68 bases between the 5' AluI site and the 5' HaeIII site. Second, the observation that both the sense and antisense 5.8S rRNA have affinity for the Y10B antibody suggest the antibody recognizes a sequence with complementary regions, which would be structurally identical when transcribed in either direction. Finally, it is possible that the epitope is not precisely defined but, rather, represents a limited structural motif (e.g., a stem–loop element) present in a variety of rRNAs. Stem–loop structures are thought to be important structural elements for protein–RNA binding (Vaughn *et al.*, 1984). For example, the structural specificity of the RNA binding site recognized by the HIV-1 TAT protein, as well as other proteins found in *Xenopus* oocytes, consists of a stem of complementary base pairs, a tripyrimidine bulge, and a six nucleotide loop (Braddock *et al.*, 1993).

Several structural elements in the proposed Y10B binding region of the 5.8S rRNA meet the previously mentioned criteria. The predicted secondary structure of the *Xenopus* 5.8S rRNA is presented in Fig. 10. Several stem–loop elements are located within the 68 bases which contain the Y10B epitope. Stem–loop structures contain runs of complementary base-pairs that form the stems. Both sense and antisense 5.8S rRNAs would contain identical stems since the complementary regions would be maintained by transcription in either direction. Alternatively, the Y10B binding site could also be a short palindrome found in a loop region (highlighted boxes in Fig. 10). This possibility is supported by the observations that nucleotides in unpaired loops are more highly conserved than base-paired regions (Vaughn *et al.*, 1984).

**Ribosome Degradation and Neuronal Death**

Several lines of evidence suggest that the subpopulation of afferent-deprived NM neurons demonstrating loss of Y10B immunoreactivity 6 h after cochlea removal eventually undergo cell death. First, the size of the Y10B-negative population 6 h after cochlea removal approximates the size of the population eventually lost from the nucleus (Garden *et al.*, 1994). Second, lack of Y10B label correlates with failed protein synthesis, which in turn correlates with EM evidence of complete ribosome destruction (Rubel *et al.*, 1991; Garden *et al.*, 1994). Third, treating animals with chloramphenicol, a mitochondrial protein synthesis inhibitor, produces a parallel increase in the size of the NM neuronal population demonstrating loss of Y10B immunoreactivity (Garden *et al.*, 1994) or ribosome degradation (Hartlage-Rübsamen *et al.*, 1992) at 6–12 h after cochlea removal, and in the number of NM neurons eventually undergoing cell death (Hyde and Durham, 1994a). The later findings that a manipulation increasing the size of the NM subpopulation demonstrating ribosome degradation and decreased Y10B immunoreactivity also increases the amount of eventual cell death.
loss strongly argues that, after 6–12 h of activity deprivation, ribosome degradation marks those NM neurons that will eventually undergo cell death. However, it is important to recognize that this population of neurons can still be saved by restoring eighth nerve activity between 6 and 12 h after the onset of deprivation. Because dying cells are marked by the structural and functional loss of cytoplasmic ribosomes prior to the commitment to the cell death process, the modification of ribosomes may be an important step in the cascade of events leading to deafferentation-induced neuronal death.

Loss of cytoplasmic ribosomes and dissociation of ribosomes from the endoplasmic reticulum of neurons undergoing cell death have been reported in several systems. Ribosome dissociation was observed during normal developmental neuronal death in chick motor neurons (Chu-Wang and Oppenheim, 1978) and rat retina (Cunningham, 1982). Target-deprived ciliary ganglia neurons also demonstrate ribosome dissociation while dying (Pilar and Landmesser, 1976), as do neurons that die during adult metamorphosis of the moth Manduca sexta (Stocker et al., 1978). Light microscopic findings suggestive of loss of rRNA (e.g., loss of Nissl stain) have also been reported in degenerating neurons of the rat cortical subplate (Al Goul and Miller, 1989). Serum-free mouse embryo (SFME) cells, a pregial cell line which is dependent on epidermal growth factor (EGF) for survival, also demonstrate ribosome dissociation as the first ultrastructural change associated with growth factor deprivation (Rawson et al., 1991).

The commonly observed picture of ribosome degradation and dissociation may be an early component of a generalized cell death program. Nuclear changes associated with the cell death process known as apoptosis eventually develop in EGF-deprived SFME cells (Rawson et al., 1991), chick motor neurons (Chu-Wang and Oppenheim, 1978), and neurons degenerating during adult metamorphosis of M. Sexta (G. Garden and J. Truman, unpublished observations). Additionally, increased RNA degradation occurs in lymphocytes just 2.5 h after glucocorticoid treatment, a traditional model of apoptosis (Cidlowski, 1982). One reviewer has even subsumed the dissociation of ribosomes and loss of polysomes from the cytoplasm into the general definition of apoptosis (Clarke, 1990). At present, it is not known whether afferent-deprived NM neurons eventually develop the nuclear changes associated with apoptosis. However, attempts to identify the chromatin cleavage characteristic of apoptosis (Wyllie et al., 1984) in NM neurons are currently underway.

Survival or Death of NM Neurons

The process of neuronal death in afferent-deprived NM neurons involves the modification of ribosome structure and function in a two-step process. Eventually, ribosome dissociation and destruction develop in the subpopulation of NM neurons which go on to die if afferent input is not restored (Rubel et al., 1991; Garden et al., 1994). However, the dramatic functional and structural alterations of ribosomes 6 h after cessation of afferent activity are reversible. Transient (6 to 12 h) blockade of afferent activity with intralabyrinthine TTX produces changes in ribosome structure and function equivalent to those seen following cochlea removal, but no neuronal loss is observed when the population is counted 1 week later (Born and Rubel, 1988; Rubel et al., 1991). This finding suggests that 6 to 12 h after loss of synaptic activity, NM neurons are not yet irreversibly committed to a cell death pathway despite the ability of markers of ribosome structure and function to identify that subpopulation, which would presumably progress to cell death, if afferent activity is not restored. Similarly, commitment to cell death occurs only after longer than 12 h of trophic factor deprivation in NGF-dependent cultured sympathetic neurons (Martin et al., 1992).

Because only a fraction of those neurons initially demonstrating loss of Y10B immunoreactivity eventually develop ribosome degradation and undergo cell death, an endogenous mechanism for reversing the initial ribosome modification must be upregulated in the surviving portion of NM neurons. Previous studies have suggested that a cell survival process, involving upregulation of oxidative metabolism, is induced by loss of afferent innervation (Hyde and Durham, 1994a, b). When this cell survival process is suppressed by treating animals with chloramphenicol, the initial loss of Y10B immunoreactivity is not reversed (Garden et al., 1994) and the majority of afferent-deprived NM neurons go on to die (Hyde and Durham, 1994a). These findings suggest that an energy-requiring process may be involved in reversing the protein–rRNA interaction that masks the Y10B epitope.

Questions and Conclusions

We have shown that the early change in ribosome structure marked by loss of Y10B immunoreactivity is secondary to protein masking of an rRNA epitope. This finding raises several questions. First, during the initial response to afferent deprivation, does a causal relationship exist between the change in ribosome structure and the functional inhibition of protein synthesis? If so, could
the molecular mechanism for the activity-dependent regulation of protein synthesis be identified by isolating the proteins involved in blocking the Y10B epitope? Second, does the protein involved in blocking the Y10B epitope have a role in the mechanism which eventually results in the death of a portion of NM neurons? Future studies aimed at identifying the molecular mechanisms responsible for altering the structure and function of ribosomes during neuronal death will aid in approaching these questions.

**EXPERIMENTAL METHODS**

**Animals**

Animals used in afferent deprivation studies were 10- to 14-day-old white Leghorn chickens. Eggs, obtained from a local supplier (H & N, Redmond, WA), were incubated and hatched at the University of Washington vivarium in AAALAC-approved facilities. Free access to food and water was available to the chicks at all times during the experiment. *Xenopus* oocytes for RNA isolation were obtained by manual extrusion from human chorionic gonadotropin-injected female frogs housed in a 64°F environment.

**Cochlea removal.** To assess the effect of afferent deprivation on the ribosomal conformation in NM neurons, chicks were subjected to unilateral removal of a cochlea. The animals were then allowed to survive for 3, 6, or 9 h following surgery. Four to five animals were assigned to each survival time. The cochlea was removed according to previously published methods (Born and Rubel, 1985). Briefly, ketamine (80 mg/kg body weight) and sodium pentobarbital (15 mg/kg body weight) were administered 5 min prior to surgery. Feathers surrounding the external ear were removed and two 5-mm incisions in the external auditory meatus were made to widen the ear canal. A pair of No. 5 Dumont forceps was used to puncture the tympanic membrane, remove the middle ear ossicle, and pull the cochlea (basilar papilla) out through the oval window. To ensure the complete receptor organ was removed, the cochlea was examined under a dissecting microscope. The middle ear cavity was then filled with gel foam and the external incisions cleaned and closed with cyano-acrylate adhesive.

**Tissue Preparation**

At the end of the designated postlesion survival period, the chicks were deeply anesthetized and then transcardially perfused with normal saline for 1–2 min, followed by 4% paraformaldehyde for 8–10 min. The heads of paraformaldehyde-perfused chicks were postfixed in 100 ml of 4% paraformaldehyde for 12 to 18 h. Fixed brain stems were dissected, blocked, thoroughly dehydrated in graded alcohols, cleared in methyl salicylate, and embedded in paraffin. Ten-micrometer serial sections were cut through the brain stem and all serial sections were retained in paraffin ribbons. Adjacent sections were mounted on alternate sets of chrome-alum-subbed glass slides. All slides were thoroughly deparaffinized prior to protease treatment or immunohistochemistry. One set of slides was labeled with standard Y10B immunohistochemistry and the second set was treated with protease prior to Y10B immunohistochemistry.

To prepare tissue for EM immunohistochemistry, 12- to 14-day-old chicks were deeply anesthetized and transcardially perfused with 0.9% saline followed by fixative (2% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The brainstem was dissected and postfixed at 4°C for 2–3 h. Fix was removed with two 10-min washes in buffer and the tissue stored in buffer overnight at 4°C. Vibratome sections (100 μm) containing NM were incubated in 0.1 M NH₄Cl for 1 h at 4°C to block free aldehydes. The NH₄Cl was removed and thick sections embedded at low temperature in Lowicryl K4M (Polysciences, Warrington, PA) using the protocol in Kiss (1989). Lowicryl-embedded tissue was flattened between sheets of acar (Ted Pella, Redding, CA) and glass slides were sealed with paraffin. The Lowicryl resin was cured with uv-light (λ = 360 nm) in a uv-cryo chamber (UVC1; Ted Pella, Redding, CA) at –5°C for 24 h. Ultrathin sections were cut on a Reichert Ultramicrotome S and collected immediately on uncoated meshed nickel grids.

**Protease treatment.** To determine if loss of Y10B immunoreactivity in afferent-deprived NM neurons resulted from protease digestible masking of the Y10B epitope, tissue was treated with Proteinase K prior to immunolabeling. The set of adjacent sections was reacted for 7.5 min at 37°C in a (20 μg/ml) Proteinase K (Promega, Madison, WI), 50 mM Tris, pH 7.5, and 5 mM EDTA solution. Proteolysis was terminated with three changes of double-distilled water. The sections were then carried through the standard Y10B immunohistochemistry procedure.

**Immunohistochemistry**

**Antibody.** Pilot studies were done using a monoclonal antibody to rRNA (Lerner et al., 1981) obtained
from Dr. Joan Steitz. Ultimately the Y10B hybridoma was obtained and cell culture supernatant which contained a high concentration of antibody was generated in cell culture facilities at the University of Washington.

**Immunolabeling procedure: Light microscopy.** All solutions in the immunohistochemistry procedure were prepared in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide. Deparaffinized sections on glass slides were rehydrated through graded alcohols and then incubated with 4% horse serum for 1 h. After this blocking step, the sections were incubated with a 1:5000 dilution of Y10B ascites or a 1:500 dilution of supernatant from cultures of the Y10B hybridoma. Incubation with the primary antibody was carried out overnight at room temperature in a humidified chamber. After three 10-min washes in PBS, sections were incubated with a 1:200 dilution of biotinylated horse anti-mouse secondary antibody (Vector Labs, Burlingame, CA) for 1 h. The secondary antibody was removed with three 10-min washes in PBS and the sections were incubated with an avidin–peroxidase conjugate (Vectastain ABC Kit, Vector Labs, Burlingame, CA) in PBS without sodium azide. After washing the sections, diaminobenzidine (0.25 mg/ml) with hydrogen peroxide (0.1%) in Tris buffer, pH 7.6, was added for 10 min. The reaction was stopped by washing the sections with PBS. To determine whether the effect of protease treatment was related to saturation of the peroxidase enzyme system, immunohistochemistry was carried out on protease-treated tissue with additional Y10B hybridoma supernatant dilutions of 1:1000, 1:5000, and 1:10,000.

**Immunolabeling procedure: Electron microscopy.** Ultrathin sections attached to nickel grids were placed face down onto droplets of blocking solution (4% normal goat serum, 1% BSA, 0.05% Triton-X in 0.05 M Tris-buffer at pH 8.2) and incubated for 1 h at room temperature in a humidified chamber. Grids were then transferred to primary antibody solution (Y10B 1:100 in 1% BSA, 0.05% Triton-X in 0.05 M Tris-buffer) and incubated overnight at 4°C. For control sections, the primary antibody solution was substituted with incubation buffer (all other parameters were the same). The grids were washed in several changes of cold 0.5 M NaCl in 0.05 M Tris-buffer (pH 8.2) and then incubated with 10–15 nm gold-particle-conjugated goat anti-mouse IgG (Ted Pella, Redding, CA) for 1 h at room temperature or overnight at 4°C. Grids were then carefully rinsed in buffer and counterstained with a saturated aqueous uranyl acetate solution for 25 min. Immunolabeled sections containing NM neurons were analyzed using a Philips 410 or a JEOL 1200 electron microscope.

**RNA Isolation**

RNA was isolated from chick brain stem or *Xenopus* oocytes by the single-step acid guanidinium thiocyanate–phenol-chloroform extraction method (Chomczynski and Sacchi, 1987).

**Immunoprecipitation**

Immunoprecipitations were done according to the methods described in Steitz (1989). Protein A–Sepharose (PAS) resin (Sigma, St. Louis, MO) was incubated with Y10B antibody overnight on a slow overhead tube rotator at 4°C. The PAS-bound Y10B resin was washed three times with 1 ml RNase-free NET-2 [150 mM NaCl, 50 mM Tris–Cl, pH 7.5, and 0.05% (v/v) Nonidet P-40]. Isolated total RNA (10 μg in 250 μl NET-2) or [35S]labeled in vitro-transcribed RNA (1 × 10⁶ cpm in 250 μl NET-2) was loaded onto the washed Y10B-PAS in the presence of 2 μl RNasin, 40 μg tRNA, and in the case of the [35S]-labeled RNA, 1 μl of 1 M diithiothreitol. This mixture was slowly rotated for 2 h at 4°C. Unbound RNA was removed by four washes with 1 ml of NET-2. After the fourth wash was removed, 300 μl NET-2, 4 μl 5 mg/ml glycogen carrier (Boehringer Mannheim, Indianapolis, IN), 30 μl 10% (w/v) sodium dodecyl sulfate, and 300 μl of SSC saturated phenol-chloroform–isoamyl alcohol (50:50:1) were added in order. The tubes were vortexed and placed in a 37°C water bath and vortexed every 5 min for 15 min. The phases were separated by spinning for 5 min in a microfuge, the aqueous phase reserved, and the RNA precipitated from the aqueous phase with ethanol. The amount of radioactivity present after immunoprecipitation of in vitro-transcribed RNA was assessed by scintillation counting.

**Electrophoresis**

**Acrylamide–Urea gels.** To visualize low molecular weight rRNAs (5S and 5.8S), immunoprecipitation products were separated on 8% acrylamide-urea gels. Immunoprecipitation products or 1 μg of total RNA were denatured in RNase-free TBE-urea sample buffer (10 M urea, 0.025% bromophenol blue, 0.025% xylene cyanol FF in TBE (90 mM Tris–Cl, 90 mM borate, and 1 mM ethylene-diaminetetraacetic acid (EDTA), pH 8.6) for 5 min at 70°C and loaded onto 7 M urea, 8% polyacrylamide TBE gel. Acrylamide gels were stained with 0.5 μg/ml ethidium bromide in TBE solution for 30 min and RNA bands visualized by exposure to high intensity ultraviolet light.
Gels loaded with radiolabeled samples were dried and exposed to X-ray film for 24 h at −70°C.

**Agarose–formaldehyde gels.** To visualize larger 18S and 28S rRNAs, immunoprecipitation products were separated on 1% agarose–formaldehyde gels. Total RNA controls or immunoprecipitation products were denaturated in 6% formaldehyde/1× Mops for 5 min at 70°C and then placed on ice. Glycerol and bromphenol blue were added to the samples and they were loaded onto a horizontal 1% agarose gel (6% formaldehyde, 1× Mops). Following electrophoresis, the agarose gels were stained for 12 h in a 0.5 µg ethidium bromide solution and visualized by exposure to ultraviolet light.

**Generation of 5.8S rRNA Probes**

**Cloning.** A cDNA which contained the entire *Xenopus* rRNA gene was obtained from the laboratory of Dr. R. Roeder. In order to generate efficient *in vitro* transcription of one of the rRNA molecules contained in this gene, we subcloned the DNA fragment encoding the 5.8S rRNA using the polymerase chain reaction (PCR). Two oligonucleotide primers were synthesized which would add an EcoRI restriction site to the 5′ end of the region coding for the 5.8S rRNA and a HindIII site at the 3′ end of the 5.8S rRNA coding sequence. PCR was carried out according to previously described methods (Schecterson and Bothwell, 1992) on the *Xenopus* rRNA cDNA. The PCR-generated 162-bp DNA fragment was inserted into pGEM3Z (Promega, Madison, WI) at the EcoRI and HindIII sites. The presence of the 162-bp fragment was confirmed in four separate clones by restriction mapping.

**In vitro transcription.** The plasmid DNA from one of the positive clones was digested with either EcoRI or HindIII to generate sense or antisense versions of the 5.8S rRNA gene. Sense 5.8S rRNA was generated using the HindIII-digested plasmid with T7 RNA polymerase and the antisense 5.8S rRNA was generated with the EcoRI-digested plasmid and SP6 RNA polymerase. *In vitro* transcription was carried out as described previously (Schecterson and Bothwell, 1992) with the following modifications to increase the amount of full-length 5.8S rRNA generated in the reaction. The concentration of cold UTP was increased from 12 to 120 μM and the enzyme reaction took place for 2 h at room temperature rather than at 37°C. To ensure that the binding of radiolabeled 5.8S rRNA to the Y10B antibody during immunoprecipitation was not being mimicked by transfer of 35S from the rRNA probe to the antibody or protein A–Sepharose, one experiment was carried out with [35P]UTP. Smaller fragments of the 5.8S rRNA (Fig. 7) were generated by restriction digests (according to enzyme manufacturer recommendations) of the plasmid with enzymes that would cut within the 5.8S gene, but would not dissociate the gene from the T7 RNA polymerase promoter.

**Data Analysis**

**Densitometry.** To quantify the relative amount of Y10B immunolabel present in afferent-deprived NM neurons compared to intact control neurons on the contralateral side of the same tissue section, the density of the DAB reaction product in each cell was analyzed using the BioQuant Image Analysis system (R & M Biometrics, Nashville, TN). Two adjacent sections from each animal, one which had been treated with Proteinase K and the other untreated prior to immunohistochemistry, were selected for analysis. Selected sections contained 50 to 80 NM neurons on each side and were from the middle (30 to 70% of the rostro-caudal axis) of the nucleus. Every NM neuron on both the afferent-deprived and control sides of the selected section was visualized under a 100× objective on a Leitz Aristoplan microscope using Nomarski optics. The outline of the cytoplasmic membrane, even in very lightly stained cells, was readily visualized. Using the outline of only the cytoplasmic portion of each NM neuron, the BioQuant system was then used to determine the optical density (OD) of immunolabel in the cytoplasm of every NM neuron in a section. To assure that the density scale (0–255) remained constant for each animal, every NM neuron in a single tissue section was analyzed at one sitting, while great care was taken to maintain a constant light level.

**Statistical analysis.** Optical density scores determined by the BioQuant program from one tissue section could not be compared to other tissue sections because of differences in the density of immunohistochemical reaction product and differences in light level. Therefore, all of the averaged OD scores for NM neurons on both sides of a section were normalized according to the following procedure. A standard score (z-score) was generated for each NM neuron by comparing the OD to the mean and standard deviation of OD scores obtained from control NM neurons of the same section according to the formula (OD − mean of the control ODs)/standard deviation of the control ODs. This formula provides a z-score which is equal to the number of standard deviations a particular OD varies from the mean OD of intact NM neurons from the contralateral nucleus in a given tissue section. Normalizing the OD of each neuron to the mean OD of control NM neurons in the same tissue sec-
tion in this manner permits comparisons between different tissue sections and different animals. In order to analyze the effect of a Proteinase K treatment on the proportion of afferent-deprived neurons which decreased in Y10B immunoreactivity, we established a cut-off point, below which a neuron was considered "Y10B negative." Neurons with a z-score of less than −2.0 were designated as Y10B negative. In other words, NM neurons with an OD less than 2 standard deviations below the mean OD of contralateral NM neurons were counted with an OD less than 2 standard deviations below the mean OD of contralateral NM neurons were counted as Y10B negative. The effect of protease treatment and afferent deprivation on raw ODs, OD z-scores, and the percentage Y10B-negative neurons at each survival time was analyzed by 2-factor ANOVA using the SuperANOVA (Abacus Concepts, Berkeley, CA) statistical analysis program. For all statistical analyses a single value was calculated for each side of the brainstem (with and without protease treatment) for each animal by averaging the raw OD scores or normalized OD scores from the 50–80 NM neurons analyzed. These values were then used for the ANOVAs and individual comparisons. A within-subject design was used for comparisons involving two sides of the same tissue section. In addition, the raw OD scores for each animal were analyzed and yielded conclusions identical to this conservative method of analyzing the group data.

**Nucleic acid analysis.** To create a model of the 5.8S rRNA secondary structure, the optimal RNA secondary structure was predicted using the MulFold RNA analysis program for Macintosh (Public domain software, Biocomputing Office, Indiana University, Bloomington, IN). This program predicts RNA secondary structure by free energy minimization according to methods described in Jaeger et al. (1989a, b) and Zucker (1989). The data output from this analysis was transformed into a graphic representation of the predicted structure using the Loop Viewer program for Macintosh created by D. Gilbert (Public domain software, Biocomputing Office, Indiana University, Bloomington, IN).

**ACKNOWLEDGMENTS**

This work was supported in part by a University of Washington Royalty Research Fund to M.A.B. and NIH Grants NS23343 to M.A.B. and DC00520 to E.W R. G.A.C. was supported by NIH Training Grant GM07266. Our thanks go to Dr. Joan Steitz for the gift of the Y10B hybridoma, Dr. R. Reeder for the gift of the plasmid containing the *Xenopus laevis* rRNA gene, Dr. Leslyanne Schecterson for expert assistance in the polymerase chain reaction, Paul Schwartz and Janet Clardy for photographic assistance, and Dr. Karen L. Deyerle and Dr. Donald E. Born for helpful comments on a previous version of this manuscript.

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Received for publication May 30, 1995