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Separate fractions of mRNA from *Torpedo* electric organ induce chloride channels and acetylcholine receptors in *Xenopus* oocytes

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Poly(A)$^+$ mRNA extracted from the electric organ of *Torpedo* was fractionated by sucrose density gradient centrifugation. After injection into *Xenopus* oocytes one mRNA fraction induced the appearance of chloride channels in the oocyte membrane. Many of these channels were normally open, and the ensuing chloride current kept the resting potential of injected oocytes close to the chloride equilibrium potential. When the membrane was hyperpolarized, the chloride current was reduced. A separate fraction of mRNA induced the incorporation of acetylcholine receptors into the oocyte membrane. When translated in a cell-free system this fraction directed the synthesis of the α, β, γ, and δ subunits of the acetylcholine receptor. In contrast, the mRNA fraction that induced the chloride channels caused the synthesis of the δ subunit, a very small amount of α, and no detectable β or γ subunits. This suggests that the size of the mRNA coding for the chloride channel is similar to the preponderant species of mRNA coding for the δ subunit of the acetylcholine receptor.

Key words: acetylcholine receptors/chloride channels/*Torpedo* electric organ mRNA/*Xenopus* oocytes

Introduction

We have shown previously that poly(A)$^+$ mRNA, isolated from the electric organ of *Torpedo* and injected into *Xenopus* oocytes, led to the synthesis and incorporation of functional acetylcholine (ACh) receptors in the surface membrane of the oocyte (Barnard *et al.*, 1982). The membrane channels opened by these ACh receptors are permeable mainly to sodium and potassium ions. The *Torpedo* electroplaque mRNA induced the oocyte to acquire also other channels, which kept the resting membrane potential of the oocyte close to the chloride equilibrium potential (R.Miledi and K.Sumikawa, unpublished data). The present work was done to determine the properties and ionic selectivity of these chloride channels. Moreover, the whole mRNA preparation used in the earlier experiments contained many different species of mRNA. We have therefore attempted to separate, by size sedimentation, the mRNA coding for the chloride channels from the mRNA coding for the nicotinic ACh receptor/channel complex.

Results

Fractionation of mRNA and translation in a cell-free system

The total poly(A)$^+$ mRNA from the electric organ was fractionated by sedimentation in a linear sucrose gradient. We report here the results obtained with two fractions that we have designated S1-19 and S2-15 (see Materials and methods and Figure 1A). When translated in a cell-free synthesizing system, fraction S2-15 directed the synthesis of all the four types of protein subunits which form the nicotinic ACh receptor (Figure 1B). In contrast, the mRNA species contained in fraction S1-19 directed the synthesis of ~15 times more δ subunit than fraction S2-15, while the other subunits were almost absent (Figure 1B). Densitometric scanning of the fluorogram of S1-19 showed no detectable β or γ subunits, and the α subunit corresponded to only ~3% of that in S2-15.

It could be that the δ subunit mRNA that sedimented in fraction S2-15 (close to the position of the 18S rRNA) was a contamination from the same δ mRNA present in fraction S1-19. However, this seems unlikely because fraction S2-15 was obtained by re-sedimentation, on an identical sucrose gradient, of fraction 15 from the original gradient. The re-sedimented fraction (S2-15) directed the synthesis of the δ subunit with efficiency equal to that of the original fraction. These results suggest that the δ subunit of the ACh receptor is coded by messengers of different sizes. The preponderant δ subunit mRNA is that contained in fraction S1-19 and presumably corresponds to the ~6000 nucleotide mRNA, determined by blot hybridization analysis (Noda *et al.*, 1983).

Expression of fractionated mRNA in Xenopus oocytes

Oocytes injected with mRNA fraction S2-15 responded to bath application of ACh with large membrane currents that desensitized rapidly (Figure 2C; Table I). These currents were similar to those induced by ACh in oocytes injected with total poly(A)$^+$ mRNA from electroplaques (Barnard *et al.*, 1982; Miledi *et al.*, 1982). On the other hand, the oocytes injected with fraction S1-19 gave only very small responses to ACh (Figure 2D) which, on average, were $<1\%$ of those elicited in oocytes injected with fraction S2-15 (Table I). These responses to ACh were due to activation of nicotinic ACh receptors induced by the injected mRNA. Native (non-injected) oocytes do not possess nicotinic ACh receptors, and although muscarinic ACh receptors are often present in *Xenopus* oocytes (Kusano *et al.*, 1982), these would have been blocked by the atropine (10^{-7} M) present in the bathing solutions.

Even though fraction S1-19 did not induce many ACh receptors, it caused the appearance of membrane channels which, in the absence of drugs, led to an appreciable increase in the resting membrane conductance. The oocytes injected with this mRNA fraction usually had lower resting potentials (about −30 mV) and input resistance than oocytes injected with fraction S2-15. To quantify the increase in membrane conductance, measurements were made of the clamp current required for a 10 mV step within the range −25 to −45 mV, where the conductance induced by the S1-19 mRNA was fully activated (cf. Figure 3). Non-injected oocytes showed a resting membrane conductance of 2.2 μS, which was not

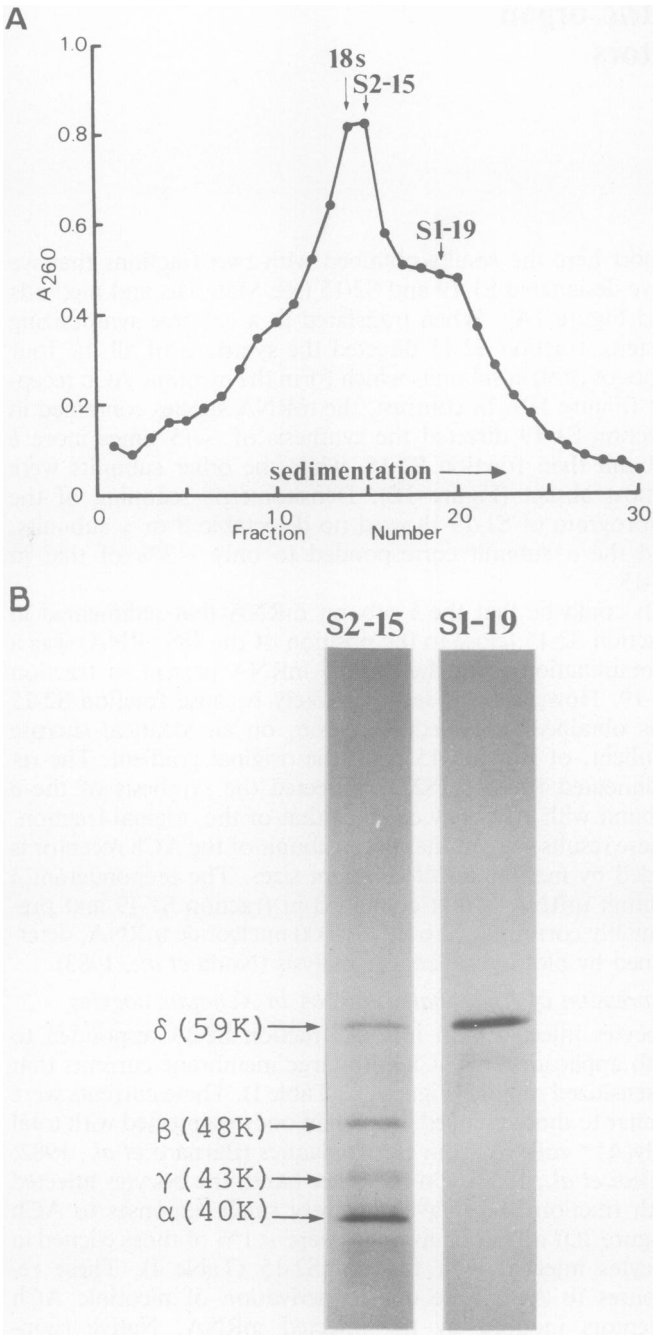

Fig. 1. (A) Sucrose density gradient centrifugation of *Torpedo* mRNA. The arrows indicate the positions of mRNA fraction S1-19, and the fraction from which S2-15 was obtained after re-centrifugation on an identical sucrose gradient. The position of the 18S rRNA sedimented in a parallel sucrose gradient is also indicated. **(B)** Electrophoretic pattern of the ACh-receptor proteins synthesized in a cell-free system. The figures show fluorograms depicting the ACh-receptor polypeptides synthesized. The positions of immunoprecipitates obtained with anti-α, β, γ and δ subunit antisera are marked (arrows) with their mol. wts. In this SDS gel system, the γ subunit synthesized in the cell-free system ran faster than the β subunit.

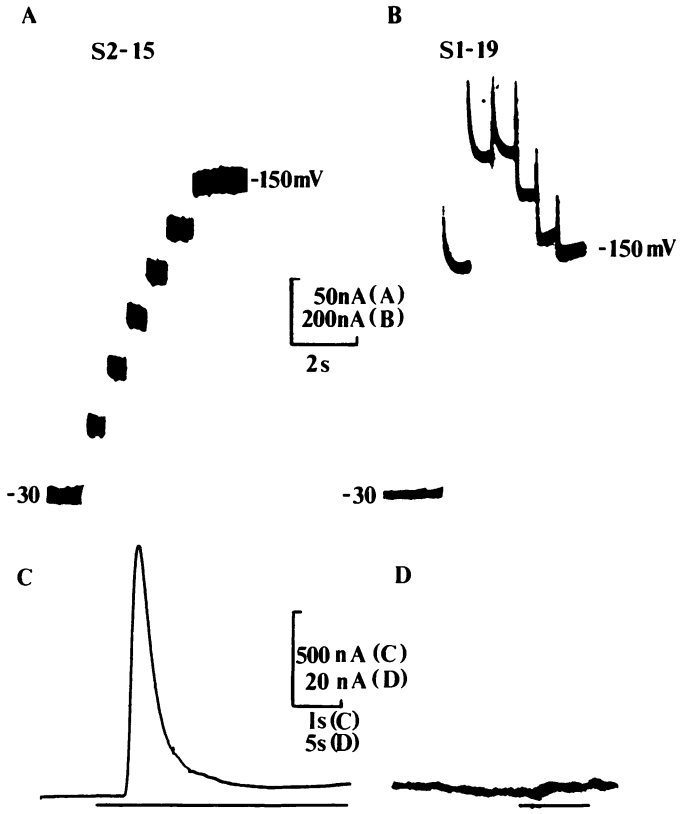

Fig. 2. ACh-activated currents and holding currents recorded in *Xenopus* oocytes injected with mRNA fractions from *Torpedo* electroplaques. The records on the left were from an oocyte injected with mRNA fraction S2-15, and those on the right from an oocyte injected with fraction S1-19. Both oocytes were from the same donor, and had been injected with mRNA 3 days previously. **(A,B)** Membrane currents elicited by stepping the membrane potential in 20 mV increments from -30 to -150 mV. Upward deflections correspond to inward membrane currents. **(C,D)** Membrane currents elicited by bath perfusion of ACh (10^{-3} M), applied for the times indicated by the bars. Clamp potential was -60 mV in **(C)** and -100 mV in **(D)**. All solutions included atropine (10^{-7} M), to block native muscarinic receptors in the oocyte membrane.

Table I. Membrane conductance and nicotinic ACh-induced currents recorded from non-injected oocytes and from oocytes injected with mRNA fractions derived from *Torpedo* electroplaques

	Conductance (μS)	ACh current (nA)
Control	2.2 ± 0.3 (6)	None detected
S1-19 mRNA	33.7 ± 5.2 (7)	20 ± 6 (12)
S2-15 mRNA	1.8 ± 0.4 (6)	2640 ± 950 (10)

Figures indicate mean ± 1 standard error of mean, with number of oocytes in brackets. All oocytes from one donor. The membrane conductance was calculated from the currents required to displace the membrane potential by 10 mV, within the range -25 to -45 mV. ACh responses were measured as the peak current elicited by 10^{-3} M ACh at a clamp potential of -60 mV. Atropine (10^{-7} M) was used to block possible native muscarinic responses to ACh.

significantly different from that of oocytes injected with fraction S2-15. In contrast, the membrane conductance of oocytes injected with fraction S1-19 was >10 times larger (Table I). Injection of the whole poly(A)$^+$ mRNA, from which the fractions derived, induced the appearance of both nicotinic ACh receptors and the increased resting membrane conductance. Since the experiments were made on oocytes treated with collagenase to remove follicular and other enveloping cells (Kusano *et al.*, 1982), the conductance increase and the ACh-sensitivity clearly reside in the oocyte membrane proper.

Properties of the membrane channels induced by mRNA fraction S1-19

To examine the voltage dependence of the conductance induced by mRNA fraction S1-19, we measured the current required to clamp the membrane potential to various levels.

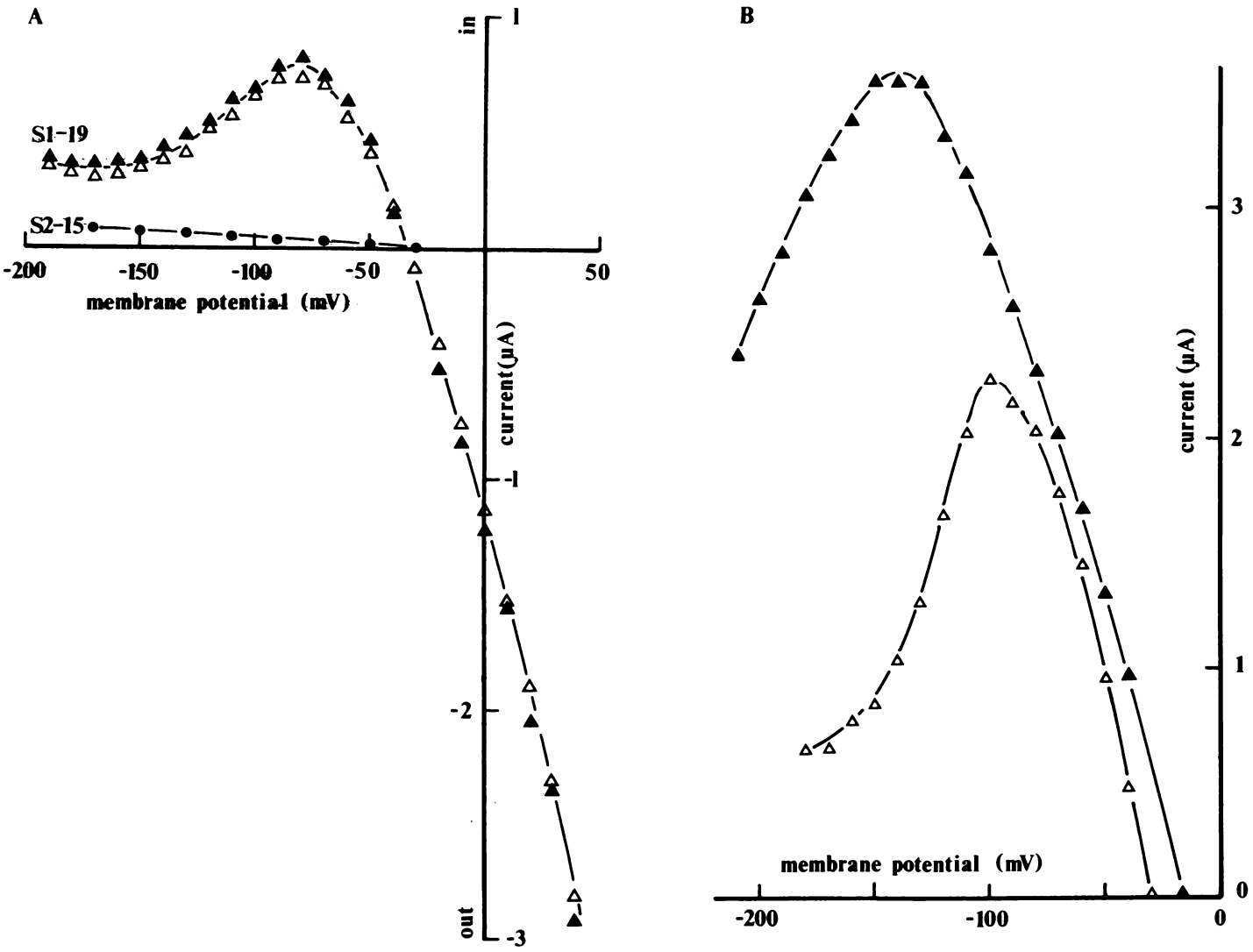

Fig. 3. Current/voltage relationships of oocytes injected with mRNA fractions from *Torpedo* electroplaques. Measurements were made by holding the membrane potential at −30 mV, and recording the currents during 3 s duration pulses to various voltages. **(A)** Full circles indicate measurements from an oocyte injected with mRNA fraction S2-15. Triangles from an oocyte injected with mRNA fraction S1-19 and bathed in normal Ringer (open symbols), or in Ringer including 40 mM K^+ (filled symbols). **(B)** Measurements from another oocyte injected with fraction S1-19, obtained in normal Ringer (open symbols), and in Ringer where one half of the NaCl was replaced by Na acetate (filled symbols).

In control oocytes, or oocytes injected with fraction S2-15, these currents were comparatively small, and decreased slightly as the inside of the oocyte was made increasingly negative (−30 to −150 mV in Figure 2A). In oocytes injected with fraction S1-19 the picture was very different. For instance, when the membrane potential was displaced from −30 to −50 mv, there was an immediate large increase in clamp current, followed by a rapid and partial subsidence, giving a 'spiky' appearance to the current step (Figure 2B). A similar, but smaller current was obtained when the membrane was hyperpolarized further, until a level was reached at which further steps gave an immediate increase in inward current followed by a rapid decline to a lower value than required to maintain the previous potential (cf. steps to −90 and to −150 mV in Figure 2B).

The current/voltage relationship measured in normal Ringer from oocytes injected with mRNA fraction S1-19 is illustrated in Figure 3 (open symbols). Over the approximate voltage range +30 to −50 mV the current varied steeply with potential, reached a maximum at around −80 to −100 mV, and decreased at more negative voltages. Similar behaviour was seeen in all oocytes injected with this fraction, while control oocytes, or oocytes injected with fraction S2-15, showed a roughly linear and much less steep current/voltage relationship (Figure 3A, circles).

The membrane current in oocytes injected with fraction S1-19 reduced to zero at a potential of -29.4 ± 1.6 mV (nine oocytes; s.e. of mean). This is close to the chloride equilibrium potential in *Xenopus* oocytes (Kusano *et al.*, 1982; Barish, 1983) and suggests that the channels induced by the messenger are permeable mainly to chloride ions. Further evidence supporting this conclusion is: (i) the current/voltage relationship was unchanged by addition of 40 mM K^+ to the Ringer solution (Figure 3a), (ii) reduction of the Cl^- concentration to one half (by substitution with acetate) shifted the reversal potential to more positive values, and increased the peak amplitude of the current at negative potentials (Figure 3B), and (iii) complete substitution of NaCl in the bathing solution by tetraethylammonium chloride caused little change in the current/voltage relationship. Thus, it seems that frac-

tion S1-19 induces the appearance of a chloride-selective conductance, which is activated at potentials more positive than about −50 mV, but which decreases progressively at more negative potentials. The activation of this conductance does not appear to involve extracellular or intracellular calcium ions, since it was little altered by intracellular injection of large amounts of the calcium chelating agent EGTA, or by addition of 10 mM manganese to the bathing solution.

Discussion

The present results show that injection of fractionated mRNA from *Torpedo* electroplaques into *Xenopus* oocytes induces the appearance of at least two types of membrane channels; a sodium/potassium selective channel opened by ACh (see also Barnard *et al.*, 1982; Miledi *et al.*, 1982) and a voltage-dependent chloride channel. Our results show also that the messengers which induce these two channels can be separated almost completely by fractionation of the mRNA on a sucrose gradient. Most probably, the messengers are translated by the oocyte into proteins which are incorporated into the membrane, where they form functional channels. However, we cannot at present rule out the possibility that latent chloride channels were already present in the native oocyte, and that the injection of mRNA fraction S1-19 in some way 'uncovered' these channels.

Native oocytes have chloride channels that are opened by an influx of calcium ions that occurs when the membrane is depolarized (Miledi, 1982; Barish, 1983; Miledi and Parker, 1984). Moreover, we have recently found that intracellular injection of calcium activates many more chloride channels than are opened during depolarization (Miledi and Parker, 1984). Therefore, we had to consider the possibility that the S1-19 mRNA had induced calcium channels in the membrane, or in some other way had increased the level of intracellular calcium, so as to cause the chloride channels to open. This does not seem to be the case because intracellular injection of EGTA, and consequent chelation of calcium, did not abolish the chloride current induced by the S1-19 mRNA, even though it does abolish the chloride current activated by membrane depolarization.

The non-innervated face of the electroplaque contains chloride channels, which have been well studied after incorporation into lipid bilayers (White and Miller, 1979; Miller, 1983); although little is known of their properties in the native cell. However, a marked difference exists between the channels incorporated into the bilayer, and those induced by mRNA. In the oocytes, the conductance is large at 0 mV, and decreases with hyperpolarization, whilst in the bilayer the chloride conductance is small at 0 mV, and increases as the *cis* face of the bilayer (to which vesicles from the electroplaques was added) is made more negative (White and Miller, 1979).

It was recently reported that the messengers of all four subunits are required to form functional ACh receptors in the oocyte (Mishina *et al.*, 1984). Our fraction S1-19, which in the cell-free system produced mainly the δ, very little α and no detectable β and γ subunits, still led to the incorporation of some ACh receptors in the oocyte membrane. The reason for this is not yet clear. Several possibilities need to be explored, including the possibility that fraction S1-19 contains sufficient α, β, and γ messengers to account for the receptors produced in the oocyte, but that these messengers are not well translated in the cell free system. The functional ACh receptors induced by fraction S1-19 were blocked by curare and by α-bungarotoxin, which suggests that they contain the α subunit.

Finally, our results suggest that the mRNA coding for the δ subunit of the ACh receptor exists in various sizes. If all four subunits are invariably required to form functional ACh receptors then, because fraction S2-15 produced many receptors, it seems likely that the smaller size of δ subunit mRNA (~18S) is involved in the production of functional ACh receptors; but the role of the larger δ subunit mRNA found in fraction S1-19 is not clear.

Materials and methods

Poly(A)$^+$ mRNA was extracted from the electric organ of *Torpedo*, using methods previously described (Miledi and Sumikawa, 1982; Sumikawa *et al.*, 1981). About 150 μg of mRNA was sedimented on a 10−31% (w/w) sucrose gradient (Sumikawa *et al.*, 1982); centrifuged for 20 h at 2°C on a Hitachi RPS 40 T rotor at 39 000 r.p.m. Fractions (~0.4 ml) were collected, precipitated with ethanol and dissolved in 20 μl of water. The fraction designated S1-19 was obtained directly from this gradient, while fraction S2-15 was obtained after re-sedimenting fraction 15 on a second (identical) gradient. A cell-free system was used to determine the ACh receptor proteins coded by these messengers. Fractions S1-19 and S2-15 were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Sumikawa *et al.*, 1981), immunoprecipitated with a mixture of ACh-receptor subunit specific antibodies (Anderson and Blobel, 1981) and electrophoresed through a 10% (w/v) SDS-polyacrylamide gel (Sumikawa *et al.*, 1981).

Methods for injection of mRNA and elecrophysiological recording from oocytes under voltage clamp were as used previously (Barnard *et al.*, 1982; Kusano *et al.*, 1982; Miledi, 1982; Miledi and Sumikawa, 1982).

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