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Selective and Rapid Nuclear Translocation of a c-Myc-Containing Complex after Fertilization of *Xenopus laevis* Eggs

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We report here unusual features of c-Myc specific to early embryonic development in *Xenopus laevis*, a period characterized by generalized transcriptional quiescence and rapid biphasic cell cycles. Two c-Myc protein forms, p61 and p64, are present in large amounts in the oocyte as well as during early development. In contrast, only p64 c-Myc is present in *Xenopus* somatic cells. p61 c-Myc is the direct translation product from both endogenous c-*myc* mRNAs and c-*myc* recombinant DNA. It is converted to the p64 c-Myc form after introduction into an egg extract, in the presence of phosphatase inhibitors. p61 and p64 belong to two distinct complexes localized in the cytoplasm of the oocyte. A 15S complex contains p64 c-Myc, and a 17.4S complex contains p61 c-Myc. Fertilization triggers the selective and total entry of only p64 c-Myc into the nucleus. This translocation occurs in a nonprogressive manner and is completed during the first cell cycles. This phenomenon results in an exceptionally high level of c-Myc in the nucleus, which returns to a somatic cell-like level only at the end of the blastulation period. During early development, when the entire embryonic genome is transcriptionally inactive, c-Myc does not exhibit a DNA binding activity with Max. Moreover, embryonic nuclei not only prevent the formation of c-Myc/Max complexes but also dissociate such preformed complexes. These peculiar aspects of c-Myc behavior suggest a function that could be linked to the rapid DNA replication cycles occurring during the early cell cycles rather than a function involving transcriptional activity.

A great deal of evidence has accumulated indicating a role for the nuclear protein c-Myc in a variety of cellular processes such as proliferation, mitogenesis, differentiation, and apoptosis (21, 50, 51). c-Myc is required for normal cell cycle progression (64, 68). Cells that express high levels of c-Myc generally exhibit reduced requirements for growth factors (2, 43, 70, 71) and exhibit higher growth rates (43, 59), characterized by a shortened G_1 phase (35). The expression of c-Myc can also overcome growth arrest (2, 41). In contrast, exit from the cell cycle or entry into the differentiation process requires down-regulation of c-Myc expression (30, 32, 62, 70). Recently a possible role for c-Myc during the G_2 phase of the cell cycle has also been suggested (67, 68).

At the molecular level, most recent evidence indicates that c-Myc is directly involved in the regulation of transcription. The c-*myc* gene encodes nuclear phosphoproteins containing three distinct domains. The COOH terminus includes a basic region followed by a helix-loop-helix leucine zipper (bHLH-Zip) domain, involved in sequence-specific DNA binding and protein-protein interaction, respectively (5). The NH_2-terminal domain of c-Myc is required for transformation (73) and has been shown to function as a transcriptional activation domain (36) able to contact the TATA-binding protein (29). Heterodimers of c-Myc and the bHLH-Zip protein Max specifically bind to the E-box sequence -CAC(G/A)TG- (4, 6, 37, 61) and can transactivate reporter gene constructs containing these binding sites (1, 42). The central domain contains a region involved in nonspecific DNA interaction (9) and a motif which confers nuclear targeting (8).

A number of indirect observations also suggest that c-Myc may be involved in the regulation of initiation of DNA replication (50, 51). Although such a function remains controversial, it may be in accordance with the growing evidence indicating that some transcription factors are also involved in the control of initiation of DNA replication (11, 31).

The early developmental period of *Xenopus laevis* provides a useful system for the analysis of c-Myc function. During oogenesis, c-*myc* is highly expressed at both the RNA and protein levels (27, 39, 75). Although the oocyte is highly active in transcription, the large store of c-Myc is entirely localized in the cytoplasm. After fertilization, the egg undergoes a series of 12 rapid cell cycles when successive S and M phases occur without G_1 or G_2 periods. During this developmental stage, the entire embryonic genome is transcriptionally silent. The c-Myc protein migrates into the embryonic nuclei during this period (27). We report here a new series of observations which show that two distinct p64 and p61 c-Myc proteins are present in the oocyte and that p64 c-Myc derives from p61 c-Myc by phosphorylation events. The two proteins are engaged respectively in two defined 15 and 17.4S complexes during oogenesis. Only p64 c-Myc and its associated complex translocate into the nuclei after fertilization, in a relatively abrupt fashion. We further show that during early development, c-Myc is unable to interact functionally with Max and to bind to the Myc/Max consensus element, suggesting that its biological role during this period does not involve such an interaction.

MATERIALS AND METHODS

Cells and embryos. *X. laevis* kidney cell line A6 was cultured at 22°C in 75% L15 medium (Gibco) supplemented with glutamine, penicillin, streptomycin, and 10% heat-inactivated fetal calf serum.

Sf9 cells cultured in TC100 insect medium (GIBCO) in suspension were infected with a baculovirus recombinant for *Xenopus* c-*myc* for expression of the protein (45).

Embryonic stages are designated as specified by Nieuwkoop and Faber (57). Collection of oocytes, eggs, and embryos and extraction of total protein were carried out as previously described (75). For oocytes, cytoplasmic and germinal vesicle extracts were obtained after manual enucleation (26).

Cytoplasmic and nuclear fractionation. Eggs and embryos were dejellied in 2% cysteine HCl (pH 7.9) and then incubated in 0.1× Barth's medium at 23°C. For each stage analyzed, embryos were pretreated by incubation in 0.1× Barth's

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medium containing 150 μg of cycloheximide per ml. After 1 h at 23°C, nuclear and cytoplasmic fractions were prepared by variations of previously described protocols (10, 27) as follows. First, a low-speed extract was prepared by centrifugational crushing for 10 min at 14,000 × *g* and 4°C. This crude extract, which contained about 80% of the original nuclei, was then used for the preparation of nuclear or cytoplasmic fractions.

For the nuclear preparation, the extract was diluted 10-fold in 20 mM potassium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6)–2% sucrose–10 mM KCl–1.5 mM $MgCl_2$–0.2 mM EDTA–0.5 mM dithiothreitol–5 μg of leupeptin per ml–5 μg of pepstatin per ml (N buffer). Nuclei were pelleted by an 8-min centrifugation at 5,000 × *g* and 4°C. The nuclei were further purified through a 0.8 M sucrose cushion in N buffer. Purified nuclei were counted after Hoechst staining of an aliquot and extracted for 45 min at 4°C in 20 mM HEPES (pH 7.6)–2% sucrose–150 mM KCl–1.5 mM $MgCl_2$–0.2% EDTA–0.5 mM dithiothreitol–0.5% Nonidet P-40–5 μg of leupeptin per ml–5 μg of pepstatin per ml (E buffer). Insoluble material was removed by centrifugation at 14,000 × *g* for 10 min at 4°C to yield the nuclear extract. No c-Myc was found remaining in the insoluble material (data not shown).

To prepare the cytoplasmic extract, the low-speed extract was diluted fivefold in E buffer without Nonidet P-40 and immediately centrifuged at 14,000 × *g* for 10 min at 4°C to pellet the nuclei. The cytoplasmic supernatant was diluted with an equal volume of E buffer with 1% Nonidet P-40 and gently mixed for 45 min at 4°C. The supernatant collected after centrifugation at 14,000 × *g* for 10 min at 4°C was the cytoplasmic extract.

Hybrid-selected translation. Hybrid selection of endogenous c-*myc* mRNAs was carried out with an M13 single-stranded *Xenopus* c-*myc* cDNA fixed on nitrocellulose filters, boiled twice in H_2O for 1 min to remove DNA not tightly bound, and hybridized by using oocyte poly(A)$^+$ mRNA. The filters were washed, and the hybridized mRNA was eluted by boiling for 1 min in water containing calf liver tRNA. RNA was recovered by ethanol precipitation, treated with RNase-free DNase (Miles), and translated in a rabbit reticulocyte lysate (Promega). The [^{35}S]methionine-labeled translation products were characterized by polyacrylamide gel electrophoresis (PAGE).

Egg extract kinase assay. Low-speed egg extracts were prepared by crushing centrifugation at 14,000 × *g* for 10 min at 2°C without dilution (7). In vitro translation of a vector containing the entire *Xenopus* cDNA sequence was carried out with the TNT kit (Promega) as instructed by the manufacturer. The expressed c-Myc protein was then incubated in the low-speed egg extract for 30 min at 23°C with or without either phosphatase inhibitors (50 mM NaF, 10 mM $NaPP_i$, 2 mg of α-naphthyl phosphate per ml, 10 mM *p*-nitrophenylphosphate, 0.1 mM sodium orthovanadate, and 1 μM okadaic acid) or kinase inhibitor (5 mM 6-dimethylaminopurine [DMAP]). Modifications of the [^{35}S]Met protein products were analyzed by sodium dodecyl sulfate (SDS)-PAGE followed by autoradiography.

E-box DNA binding assays. Protein extracts were incubated for 15 min at 30°C with or without addition of glutathione *S*-transferase (GST)–Max151, a fusion protein containing the whole coding sequence of human Max linked to the GST peptide (4). A preformed complex, GST-Max151/GST-MycC92, was also assayed; GST-MycC92 was a fusion protein containing the 92 C-terminal amino acids of the *Xenopus* c-Myc sequence linked to the GST peptide. The E-box double-stranded oligonucleotide was ^{32}P labeled and added to the extract for 30 min at 4°C before sedimentation through sucrose gradients.

Sucrose gradient analysis. Protein extract (0.2 ml) was layered over a 5-ml linear 5 to 20% sucrose gradient in E buffer, layered over a 60% sucrose pad (0.3 ml). Centrifugation was in a Beckman TST 55.5 rotor at 40,000 rpm and 4°C. Centrifugation was for 14.5 h to resolve fractions in the 4 to 10S range or at 55,000 rpm for 2 h 45 min for fractions in the 10 to 20S range. Fractions of 0.180 ml were collected, and aliquots of each fraction were analyzed by immunoblotting.

Immunodetection and quantification. The anti-*Xenopus* c-Myc polyclonal antibody was used in conditions previously described (46). The anti-Max (human) serum was kindly provided by E. Ziff and did not react with the *Xenopus* Max proteins. The anti-GST antibody used to revealed fusions GST-Max151 and GST-MycC92 together was provided by J. C. Courvalin. Intensity of the signals generated on the blots was measured with a Schimadzu model CS-930 dual-wavelength thin-layer chromatoscanner.

RESULTS

Developmental regulation of the maternal c-Myc store. Two distinct c-*myc* mRNA classes have been identified in *X. laevis*, possibly expressed from the duplicated c-*myc* locus (39, 75, 77). Two c-Myc proteins, p61 and p64, have also been found in oocytes, but it was not clear whether each protein arose from a specific mRNA class (38, 39) or was a consequence of the pseudo-tetraploid nature of the *Xenopus* genome (3) or whether the two proteins were related to each other by post-translational modifications. To check if the two proteins were derived from two distinct mRNAs, we isolated the endogenous

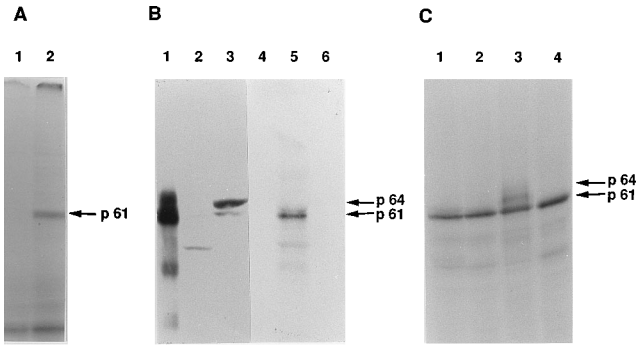

FIG. 1. Origin of the two c-Myc forms p61 and p64. (A) In vitro translation of hybrid-selected oocyte c-*myc* mRNA in a reticulocyte cell-free system was as described in Materials and Methods. Translation products were analyzed by SDS-PAGE followed by fluorography. Lane 1, background synthesis without addition of exogenous RNA; lane 2, translation products of hybrid selected c-*myc* RNAs. (B) c-Myc proteins produced either from insect cells infected with a recombinant *Xenopus* c-*myc* baculovirus (lane 1), from c-*myc* mRNA translated in a reticulocyte cell-free system in the presence of [^{35}S]Met (lane 2), or from *Xenopus* oocytes (lane 3) were electrophoresed on the same gel and detected by immunoblotting with a *Xenopus* c-Myc antibody. Although a large amount of reticulocyte lysate was loaded, the amount of ^{35}S-labeled c-Myc protein produced (<1 ng) did not permit its detection, and thus the same blot was also submitted to autoradiography (lanes 4 to 6). Lane 5 corresponds to lane 2 and shows the position of ^{35}S-labeled c-Myc protein. Lanes 4 and 6 gave no signal, as they correspond to lanes 1 and 3 (same blot). (C) c-Myc protein was translated from *Xenopus* c-*myc* in reticulocyte lysate (lane 1); 1.5 μl of the lysate was then mixed in 3.5 μl of *Xenopus* egg extract for 30 min at 23°C in the absence (lane 2) or presence (lane 3) of phosphatase inhibitors or in presence of the generalized kinase inhibitor DMAP (lane 4).

oocyte c-*myc* mRNAs by hybrid selection with a single-stranded c-*myc* DNA probe (Materials and Methods). The hybridized mRNAs were eluted from DNA, and the corresponding protein products were identified by in vitro translation in a reticulocyte lysate. As show in Fig. 1A, the hybrid-selected mRNAs encode a unique protein product of 61 kDa.

We then compared the apparent molecular weights of the proteins synthesized either in vivo in oocytes, from c-*myc* mRNA translated in a reticulocyte lysate, or from a *Xenopus* c-*myc* baculovirus vector expressed in insect cells (Materials and Methods). Figure 1B shows this comparison on a blot which was analyzed both by immunodetection with a c-Myc antibody (lanes 1 to 3) and by autoradiography (lanes 4 to 6). Changes in c-Myc apparent size could then be detected under identical electrophoresis and blotting conditions. The endogenous oocyte c-Myc protein migrated as two distinct forms, p61 and p64 (Fig. 1B, lane 3). p64 c-Myc is always the major form observed, although the precise ratio of the two forms can vary slightly with the conditions of preparation. The reticulocyte translation product migrated as a single ^{35}S-labeled p61 c-Myc protein, which was also the major band observed in the baculovirus-expressed protein (compare lanes 1 and 5). Upper bands with apparent molecular masses of about 64 kDa were also observed in the baculovirus-expressed proteins (lane 1), and since baculovirus-expressed proteins are subjected to eukaryotic posttranslational modifications, these bands could correspond to different phosphorylation states of c-Myc (51). To assay if p64 c-Myc could derive from the p61 c-Myc polypeptide by phosphorylation occurring in the egg, we incubated the p61 c-Myc product previously synthesized in reticulocyte lysate in an egg extract (Fig. 1C). A shift from p61 to p64 c-Myc was indeed observed when phosphatase inhibitors were present (Fig. 1C, lane 3), consistent with a kinase activity present in the egg extract which was able to generate the p64 c-Myc species. Accordingly, the egg extract failed to convert

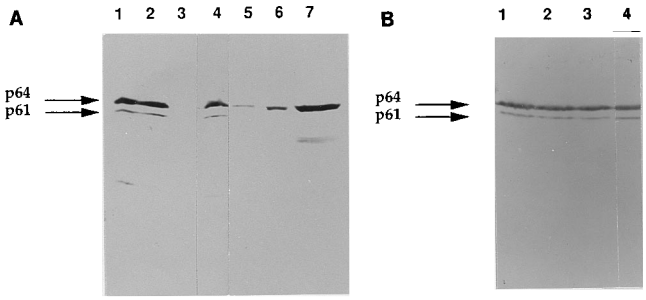

FIG. 2. Expression of c-Myc protein during early development. Total protein extracts from various developmental stages as well as somatic cells were analyzed by immunoblotting. (A) Extracts of one oocyte (lane 1), one oocyte cytoplasm (lane 2), one oocyte nucleus (lane 3), one egg (lane 4), and 5×10^4 (lane 5), 10×10^4 (lane 6), and 50×10^4 (lane 7) *Xenopus* A6 cells in exponential phase. (B) Extracts of one oocyte (lane 1), one egg (lane 2), one 2,000-cell-stage embryo (lane 3), and one 16,000-cell-stage embryo (lane 4).

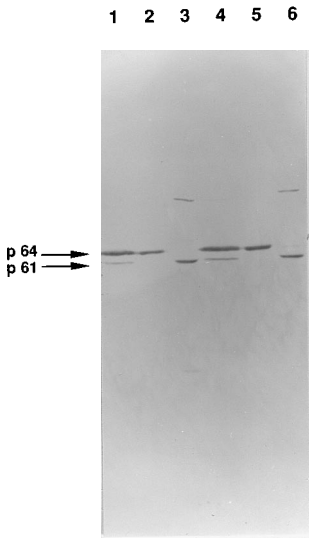

FIG. 3. Subcellular localization of c-Myc protein during early development. Total protein extracts from 2,000- and 16,000-cell-stage embryos were analyzed by immunoblotting after separation of nuclei from cytoplasm. Shown are total extract (lane 1), nuclear extract (lane 2), and cytoplasmic extract (lane 3) from 2,000-cell-stage embryos and total extract (lane 4), nuclear extract (lane 5), and cytoplasmic extract (lane 6) from 16,000-cell-stage embryos.

p61 to p64 c-Myc in the presence of the generalized kinase inhibitor DMAP and did not reveal lower-molecular-weight bands (lane 4). Interestingly, both p64 and p61 c-Myc are found in equilibrium in vivo, and we have not been able to detect any conversion of the endogenous p64 to p61 in the presence of DMAP.

The observation of a unique p61 c-Myc protein species as an in vitro translation product of the endogenous c-*myc* mRNAs, together with the conversion of this form to p64 c-Myc in the egg extract, strongly suggests that p64 and p61 are related by posttranslational modifications affecting p61 after its synthesis. Both p61 and p64 c-Myc are stored in the cytoplasm of the mature oocyte, in contrast to proliferating cells, in which c-Myc is found in the nucleus. Interestingly, only one c-Myc species, the p64 protein, was detected in *Xenopus* cells growing in culture (Fig. 2A, lanes 5 to 7). Note that the amount of p64 c-Myc found in 3×10^5 to 5×10^5 growing cells is stored in a single oocyte. The p64/p61 ratio and the total amount of c-Myc protein do not significantly vary during early development (Fig. 2B).

A detailed analysis of the nuclear migration of c-Myc induced by fertilization revealed two unexpected observations. First, only p64 c-Myc migrates into the nucleus, whereas p61 c-Myc remains in the cytoplasm of the embryos (Fig. 3). As c-Myc is a protein involved in nuclear processes, this observation suggests that p64 is the functional form of c-Myc. Second, the translocation of c-Myc protein appeared complete by the 2,000-cell stage. This observation prompted us to analyze the very early cell cycles. Embryonic nuclei were therefore purified from several stages of early development as detailed in Materials and Methods, and their c-Myc content was quantified by immunoblotting (Fig. 4B and C). The earliest stage which could be experimentally tested was the 16-cell stage. The content of 2,000 nuclei was analyzed for each stage except the 16-cell stage, in which case an extract equivalent to 10-fold-fewer nuclei was loaded on the gel to avoid a saturated signal. Figure 4A shows that the entire p64 c-Myc stored in the oocyte cytoplasm had translocated into the nuclei by the very early cell cycles, as at the 16-cell stage, p64 was undetectable in the cytoplasm (Fig. 4A, lane C; see also Fig. 7). Subsequently, this maternal store is distributed among the daughter cell nuclei during the following cell cycles. Quantification of the p64 c-Myc signal per nucleus confirms the massive entry of c-Myc into the first embryonic nuclei, followed by an exponential dilution in the daughter nuclei which was completed by the blastula/gastrula stage. We conclude that the maternal store of p64 c-Myc is not recruited as a simple structural component of the embryonic nuclei. In this case, the recruitment would be proportional to the number of dividing nuclei. Instead, the first embryonic nuclei received the whole store of p64 c-Myc, resulting in an abnormal amount of c-Myc per nucleus during this early developmental period. Interestingly at the blastula/gastrula stage, the amount of c-Myc per nucleus approaches that found in a somatic cell in culture.

p64 and p61 c-Myc are associated with two different high-molecular-mass complexes. In human somatic cells, c-Myc is found only associated with Max, and in glycerol gradients, it migrates as a low-molecular-mass complex corresponding to 70 kDa (4.4S) (49). Other studies performed with transformed cells show that c-Myc can also associate with high-molecular-mass complexes. Using cross-linking experiments, Gillespie and Eisenman (24) found a 500-kDa complex (15S), whereas Studzinski et al. (74) reported an association of c-Myc with a 600-kDa complex (around 17S). The selective and rapid nuclear entry of only one of the two c-Myc species led us to search for the possible association of c-Myc with protein complexes unique to early development.

A variety of nuclear and cytoplasmic extracts were isolated and fractionated on sucrose gradients, and positions of c-Myc within these gradients were identified by immunoblotting. Sedimentation of a nuclear extract prepared from *Xenopus* somatic cells in culture revealed two c-Myc populations; a high-molecular-weight complex involved 70% of the total c-Myc, whereas the remaining 30% sedimented as a 4.4S complex (Fig. 5). Only p64 c-Myc was detected, as expected from immunoblots of total cell extracts (Fig. 2). A rather different result was obtained with extracts from oocytes or unfertilized eggs. Most of p64 c-Myc and p61 c-Myc migrated as high-molecular-weight complexes that were unresolved under these conditions (Fig. 6A). In conditions permitting the resolution of such complexes, p64 and p61 c-Myc were found associated with two distinct, well-defined peaks sedimenting at 15 and 17.4S (Fig. 6B). Quantification showed that these complexes represented more than 98% of the total c-Myc. The mobilities of

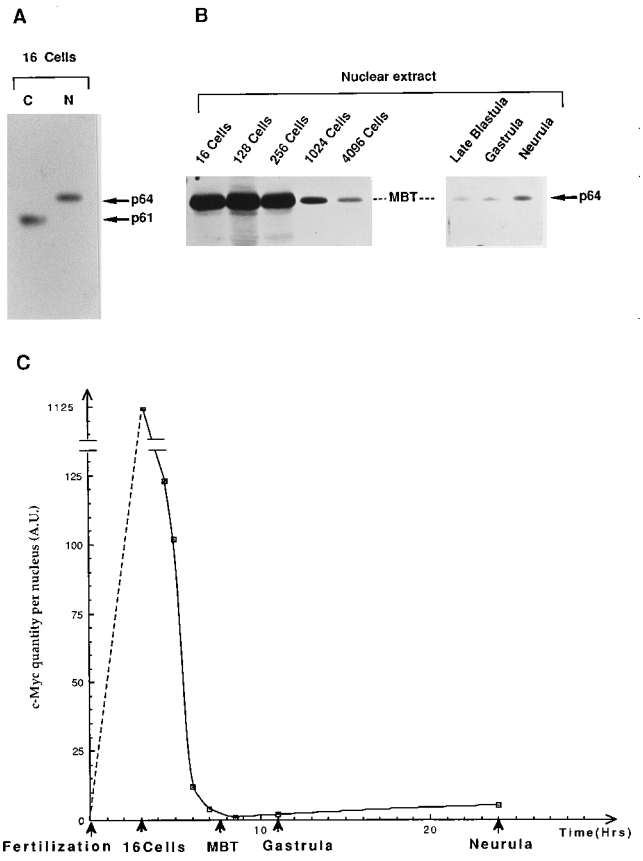

FIG. 4. Variation of nuclear p64 c-Myc during early development. (A) Nuclear (lane N) and cytoplasmic (lane C) content of a 16-cell-stage embryo analyzed by immunoblotting. (B) Nuclear protein extracts analyzed by immunoblotting. Each lane represents an extract from 2,000 purified nuclei except for the 16-cell stage, which corresponds to 200 purified nuclei. MBT, midblastula transition. (C) Amount of c-Myc per nucleus (arbitrary units [A.U.]) as a function of developmental stage. Quantification of the immunoblot (B) was performed as described in Materials and Methods.

these different complexes were unchanged after treatment with DNase and RNase (data not shown). The nearly complete absence of the 4.4S c-Myc complex in oocytes or unfertilized eggs may indicate that any function associated with this complex is not required in these cells.

Fertilization induces the entry of p64 c-Myc into the nuclei. To detect any change related to this migration, we performed a sucrose gradient analysis of the c-Myc complexes both in the cytoplasm and in the nuclei of young embryos. Figure 7 presents the results obtained at the 16-cell stage. As shown above, p64 c-Myc is the only species present in the nucleus (Fig. 7A and B), whereas p61 c-Myc remains in the cytoplasm (Fig. 7C and D). p61 c-Myc was found associated with a 17.4S complex, similar in size to the complex found in the oocyte cytoplasm and unfertilized egg (Fig. 7C and D). Similarly, the p64 c-Myc-associated complex did not change in size (15S) when localized in the nucleus after fertilization (Fig. 7A and B). Because of its large size, this 15S complex is unlikely to enter the nuclei passively but could be incorporated within the nuclei during their formation and anchored to nuclear structures segregating with the daughter nuclei during early development. In accordance with this possibility, the analysis of several stages of early embryonic development indicates that neither the size nor the localization of either c-Myc complex appears to vary from the 16-cell stage to the 16,000-cell stage (data not shown).

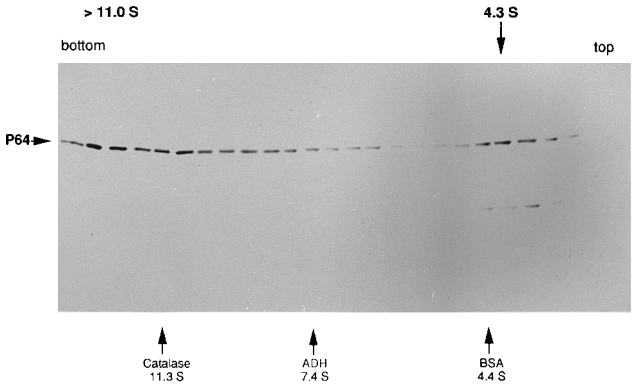

FIG. 5. c-Myc complexes from an A6 cell nuclear extract analyzed by sucrose gradient sedimentation. Protein extracts from 50×10^6 A6 nuclei were layered over a 5 to 20% sucrose gradient and centrifuged for 14.5 h at 40,000 rpm and 4°C. Arrows at the bottom indicate protein markers run in parallel. BSA, bovine serum albumin; ADH, alcohol dehydrogenase.

We conclude that fertilization does not change the physical nature of the maternal c-Myc complexes, although there is a dramatic and rapid change in the localization of one specific complex (the p64 complex). Moreover, the structure of the p64 complex does not change during the entire early developmen-

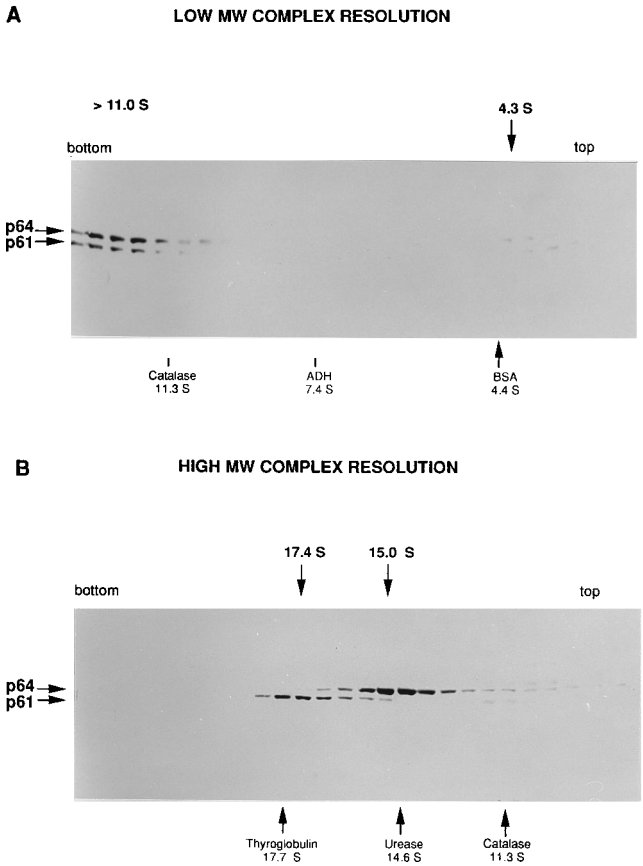

FIG. 6. c-Myc complexes from egg extracts analyzed by sucrose gradient sedimentation. Protein extracts from 100 eggs were layered over a 5 to 20% sucrose gradient and centrifuged at 4°C at 40,000 rpm for 14.5 h (A) or at 55,000 rpm at 4°C for 2 h 45 min (B). MW, molecular weight.

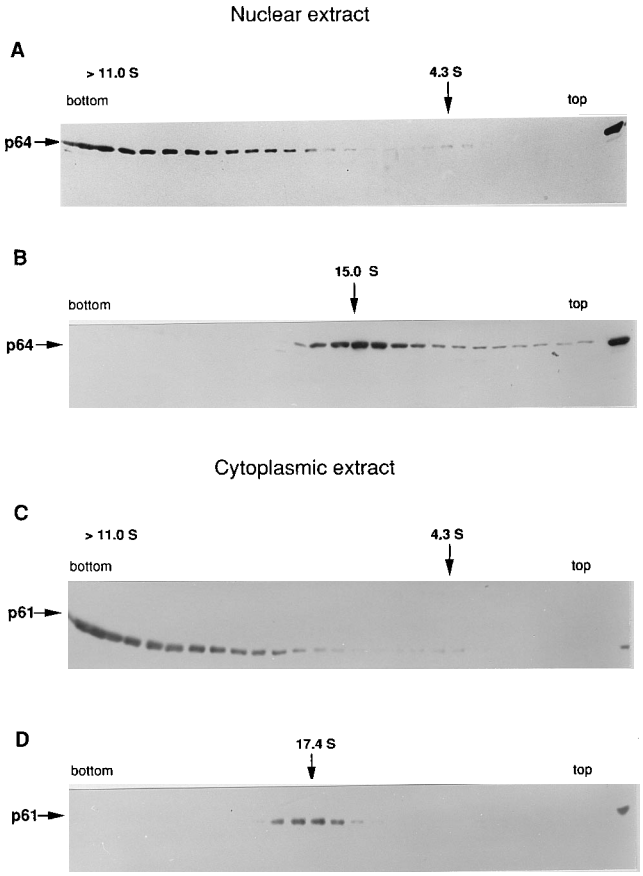

FIG. 7. c-Myc complexes in 16-cell-stage embryos. (A and B) Nuclear protein fraction from 16-cell-stage embryos. (C and D) Cytoplasmic protein fraction from 16-cell-stage embryos. Protein extracts isolated after cellular fractionation were layered over 5 to 20% sucrose gradients and centrifuged at 40,000 rpm at 4°C for 14.5 h (A and C) or 55,000 rpm at 4°C for 2 h 45 min (B and D).

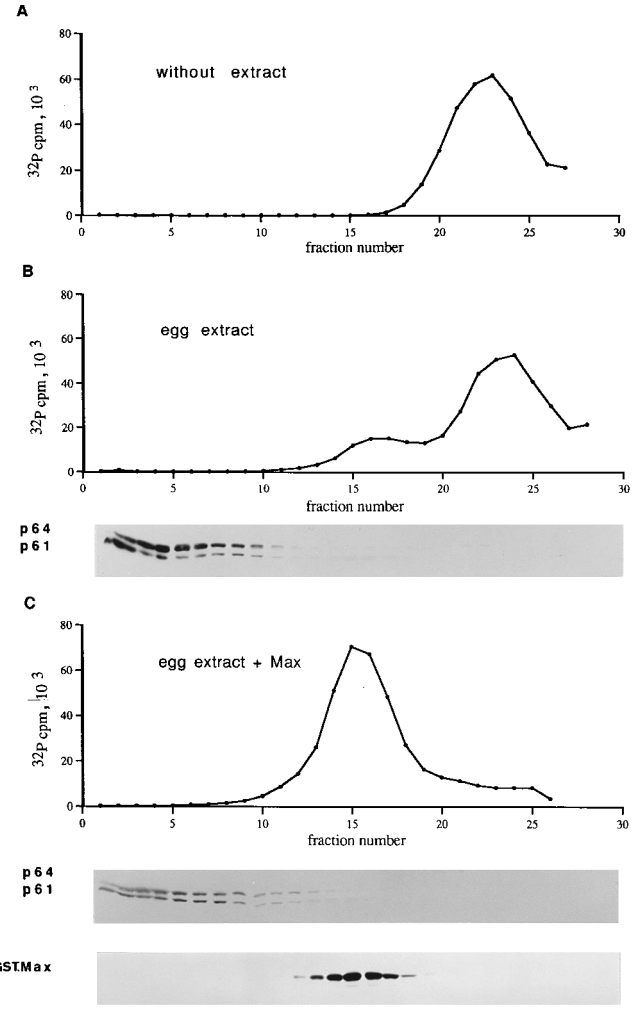

FIG. 8. c-Myc DNA binding activity in an egg extract, analyzed by sucrose gradient fractionation. An egg extract was tested for its ability to specifically bind the E-box -CACGTG- sequence without (B) or with (C) the addition of human GST-Max151 (clone provided by R. N. Eisenman). Samples were incubated for 15 min at 30°C without DNA, to allow protein exchanges. A ^{32}P-radiolabeled double-stranded oligonucleotide (-AATTCGACCACGTGGTCG-) was added, and incubation was continued for 30 min at 4°C before fractionation on a 5 to 20% sucrose gradient. The sedimentation of the free oligonucleotide, without egg extract, is shown in panel A. Gradients were centrifuged at 40,000 rpm for 14.5 h at 4°C. Each fraction was counted and tested by immunoblotting for the presence of c-Myc (B and C) and GST-Max (C).

tal period, indicating that the function associated with this complex could be established from the very earliest cell cycles.

c-Myc does not bind to the E box during early development. Several studies have clearly demonstrated that c-Myc is involved in transcriptional regulation, in association with Max, a bHLH-Zip protein. Myc and Max interact through their helix-loop-helix and leucine zipper domains (4, 6, 61) and specifically bind to the E-box sequence -CAC(G/A)TG-. Max homodimers but not c-Myc homodimers also recognize this sequence (1, 6, 49).

As the binding to the E box may reflect c-Myc transcriptional activity, we investigated if such DNA binding activity was associated with the c-Myc complexes during early development. When fractions containing the two 15 and 17.4S complexes from unfertilized eggs were analyzed by an electromobility shift assay using the -CACGTG- sequence, no DNA binding was detected (data not shown). However the size of the complex could have hampered such an analysis. We therefore assayed binding activity in the egg extract by sedimentation in sucrose gradients. The position of the labeled oligonucleotide was identified by its radioactivity, whereas the position of c-Myc was determined by immunoblotting of the corresponding sucrose gradient fractions. The free oligonucleotide migrated at the top of the gradient with or without addition of an egg extract (Fig. 8A and B). A second peak was observed with the egg extract, although neither p64 nor p61 c-Myc sedimented at this position. This result indicated that factors other than c-Myc were binding the E box.

To determine if a DNA binding activity involving Max could be observed in such gradients, the egg extract was preincubated with a recombinant human Max protein (GST-Max151) before sedimentation. This recombinant protein interacts with the E-box sequence in association with *Xenopus* c-Myc protein (45). Figure 8C shows that a GST-Max/oligonucleotide complex was obtained, at the position expected for the GST-Max homodimer. Immunoblot analysis with an antibody specific for human Max (which does not react with endogenous *Xenopus* Max) confirms its presence in this complex. No association of exogenous GST-Max was found at the position of the c-Myc complexes. We concluded that either Max is not associated with the c-Myc complexes in the egg or its association with such complexes prevents its ability to bind the E-box sequence.

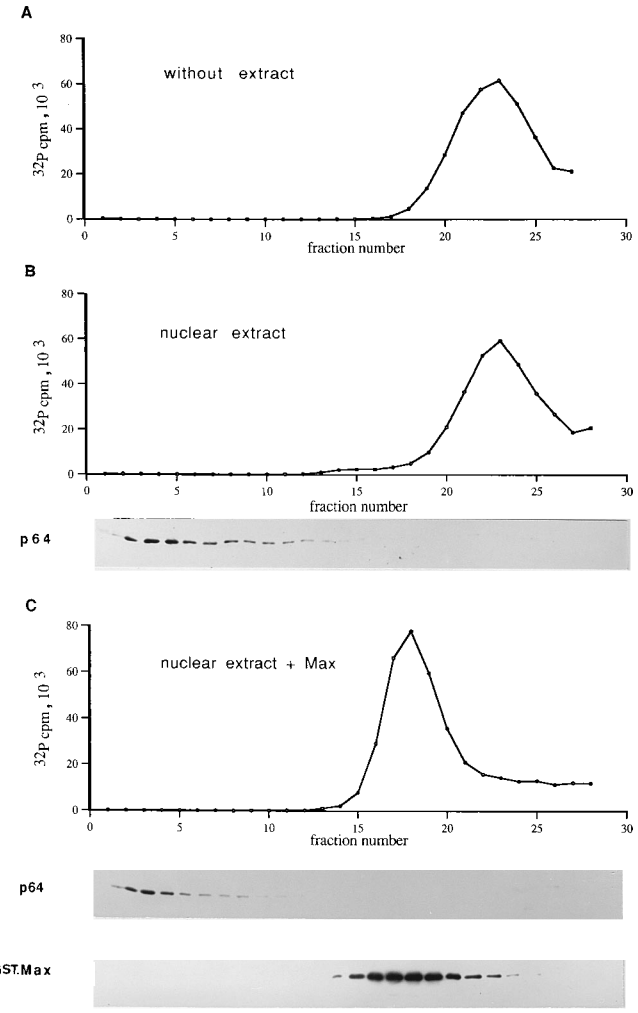

FIG. 9. c-Myc DNA binding activity in the nuclei of 16-cell-stage embryos analyzed by sucrose gradient fractionation. A nuclear extract was prepared from 16-cell-stage embryos and analyzed for the ability to specifically bind the E-box sequence, using the procedure described for Fig. 7. (A) Radiolabeled double-stranded oligonucleotide without extract. (B) Nuclear extract of 16-cell-stage embryos. (C) Nuclear extract of 16-cell-stage embryos with added GST-Max151.

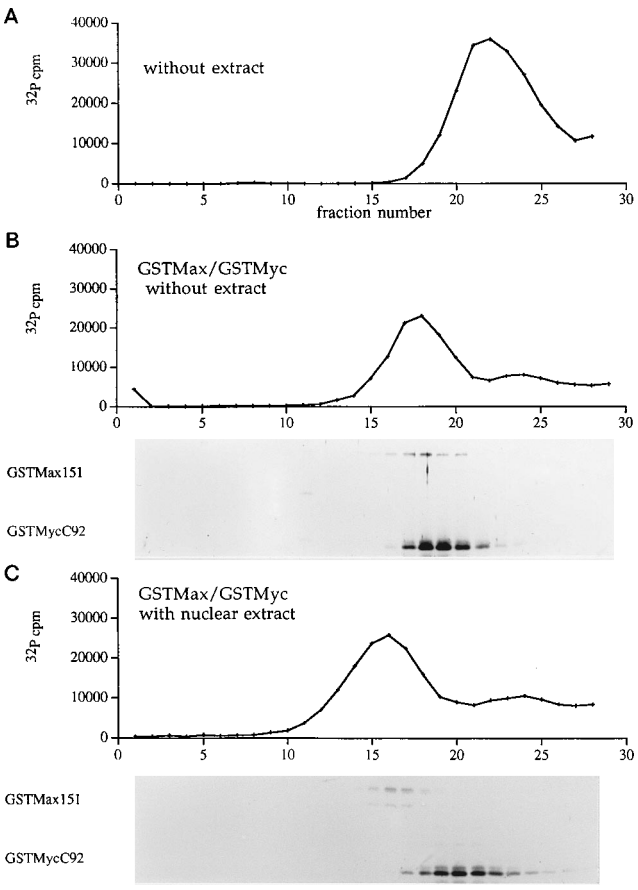

FIG. 10. Inhibitory activity of the formation c-Myc/Max heterodimers contained in the nuclei of 32-cell-stage embryos. A nuclear extract was prepared from 32-cell-stage embryos and analyzed for the ability to specifically bind the E-box sequence, using the procedure described for Fig. 7. (A) Radiolabeled double-stranded oligonucleotide without extract. (B) Specific binding of the GST-Max151/GST-MycC92 heterodimer. (C) Nuclear extract of 32-cell-stage embryos with added heterodimer GST-Max151/GST-MycC92.

A dramatic change in the localization of p64 c-Myc occurs after fertilization. We therefore assayed if the nuclear translocation could correlate with a change in the DNA binding properties. Figure 9 shows that no DNA binding activity was associated with p64 c-Myc in the nuclei during early development. Although GST-Max can bind the E-box sequence when added to the extract, it does not associate with the endogenous c-Myc. The lack of detectable c-Myc/Max DNA binding activity in the egg extract could be due to the inaccessibility of c-Myc to Max when present in the 15S complex. Alternatively, the formation of the c-Myc/Max complex could be repressed in the egg.

To analyze this possibility, we first preformed a c-MycC92/Max151 complex. Such complexes are known to be able to bind the E-box sequence (4). Analysis was performed by sucrose gradient fractionation in the same conditions as used previously. Figure 10B shows that a complex indeed formed between the two proteins, as indicated by a shift of the oligonucleotide to a position in the gradient where Myc and Max cofractionate. In contrast, the addition of the embryonic nuclei extract before fractionation on sucrose gradients induces the dissociation of the complex. Moreover, the position of the oligonucleotide is now shifted to the position of the GST-Max151 dimer, as expected for the higher molecular weight of the GST-Max151 dimer than the GST-Max151/c-MycC92 dimer (Fig. 10B and C).

We concluded that c-Myc is inactive in its DNA binding activity during early development and that it is engaged in a complex which cannot physically interact with exogenous Max. In addition, the formation of a c-Myc/Max heterodimer is inhibited in the embryo. The lack of c-Myc/Max DNA binding activity to the E-box sequence, at a time when the genome is transcriptionally quiescent, may indicate that c-Myc is involved in another process during early development.

DISCUSSION

c-Myc properties during early development contrast with those observed in somatic cells. During early development, the localization, physical properties, and activity of c-Myc strikingly differ from the previously known properties of c-Myc in somatic cells. These observations suggest that the function of c-Myc during early development is different from that in somatic cells.

Although *Xenopus* c-Myc contains a conserved nuclear lo-

calization signal (NLS) (8) and is entirely localized in the nuclei of *Xenopus* somatic cells in culture, it is stored in the cytoplasm of the oocyte. This behavior may be explained in two ways. Either the oocyte machinery is deficient in recognizing an NLS, or the c-Myc protein is sequestered in the cytoplasm by a process that interferes with NLS function. The first possibility is unlikely, as oocyte microinjection has been successfully used to analyze nuclear migration of NLS-containing proteins (13, 14, 48). Cytoplasmic retention of c-Myc in the oocyte might be due to interaction with a cytoplasmic anchor protein. Such a mechanism was observed for the *Drosophila* Dorsal protein, which is maintained in the cytoplasm in the dorsal region of the embryo through direct interaction with Cactus (63, 65, 72). Similarly, the transcription factor NF-κB is complexed with Cactus (63, 65, 72). Similarly, the transcription factor NF-κB is complexed with the protein IκB in unstimulated B lymphocytes, resulting in its sequestration in the cytoplasm and an inability to translocate to the nucleus (47, 66).

In vivo translation of c-*myc* RNAs gives rise to p61 c-Myc, which converts to a phosphorylated p64 c-Myc form after incubation in an egg extract when phosphatases are inhibited. Both p61 and p64 c-Myc are present in equilibrium in *Xenopus* oocytes and are stored in high-molecular-weight complexes that are unlikely to passively enter the nucleus, supporting a model of cytoplasmic sequestration. After fertilization, only p64 c-Myc is found in the nuclei, associated with a complex of similar size. The changes associated with oocyte maturation and egg fertilization together with the specific features of the cell cycle during early development may give some explanation for the translocation specific to the fertilized egg. During maturation of the oocyte into an egg, the nuclear membrane breaks down and is reconstituted only after fertilization. Nuclear membrane breakdown and re-formation then occur during each of the following rapid cell cycles (containing only S and M phases), using preformed structures present in the unfertilized egg (23, 56). We propose that the uptake of p64 c-Myc by the nuclei occurs during the nuclear formation in the first cell cycle. In contrast, p61 c-Myc is never found in the nuclei, either in oocytes or in fertilized embryos, and it is also absent in somatic cells. This observation strongly suggests that the phosphorylated p64 c-Myc is the functional form and leaves open the possibility of another function associated with p61 c-Myc.

An unexpected but significant finding was that no binding activity of c-Myc to the E-box sequence was detected in eggs or early embryos, even when the protein was localized in the nuclei. First, no E-box activity was recovered in sucrose gradient fractions containing c-Myc; second, we were not able to detect the formation of a c-Myc/Max complex, the active form of c-Myc in transcriptional activation (1), when exogenous Max was added to egg or embryonic nuclear extracts. Third, an in vitro-preformed c-Myc/Max complex is dissociated when incubated in an embryonic nuclei extract.

These data taken together suggest that if c-Myc is involved in a function specific for early development, it not related to Max-associated specific DNA binding.

Specific features of c-Myc during early development as a possible indication of its function. One prominent aspect of early development is the accumulation in the oocyte of a maternal store of proteins and RNAs that will be required during the early cleavage stages (10). In *X. laevis*, this accumulation is especially important because of the absence of gene expression during the first 12 cell cycles. The distribution of maternal components after fertilization is therefore critical in determining their function in the early embryo.

There are several examples of the selective transport of proteins into nuclei at the time they become functional (33, 58, 66, 69). In *X. laevis*, a series of detailed studies on the accumulation of maternal nuclear proteins in embryonic nuclei showed that two classes of proteins can be distinguished. One class accumulates in a progressive fashion in the cleavage nuclei, and the second class is excluded from the nuclei until late blastula or gastrula (15, 18, 54). In all cases, nuclear accumulation appears to be a gradual process. The rapid nuclear uptake of the p64 c-Myc-containing complex, complete from the very early cell cycles, therefore contrasts with the two classes defined above. Among the late-migrating proteins are proteins that bind to small nuclear U RNAs. These proteins remain in the cytoplasm of the early embryo until the onset of transcription at the midblastula transition (12, 79), when they enter the nuclei. Likewise, Xnf7, a putative transcription factor enriched in nuclei of the central nervous system, is maintained in the cytoplasm of the early embryo and accumulates in nuclei only by the midblastula to gastrula stage (16, 53). Of the early-migrating proteins described (17), N1, N2, and nucleoplasmin are involved in chromatin assembly (13, 40, 44), and lamin LIII is a structural component of the nuclear envelope. The progressive migration of these proteins into the embryonic nuclei during the cleavage stages obviously correlates with their known functions in nuclear metabolism. The rapid entry of p64 c-Myc and its associated complex into nuclei during early development may thus suggest a specific function which must be established soon after fertilization.

During oogenesis, a period of active transcription, c-Myc is present in the cytoplasm. This localization makes unlikely the participation of c-Myc in transcriptional processes during this period. After fertilization, cells are actively engaged in DNA replication, and during this period no transcription is observed (25, 55). The developmental regulation of c-Myc and its unusual translocation suggest a role in DNA replication. When replication is not occurring, c-Myc is stored in the cytoplasm, whereas it migrates to the nucleus as soon as cell division is induced by fertilization. At this point, the level of p64 c-Myc per genome is 3×10^5-fold the level in a somatic cell. The machinery for initiating DNA replication is already present in unfertilized *Xenopus* eggs and can replicate large amounts of injected DNA (7, 23, 28, 52). Moreover, during early development, an increase in nondefined replication origins permits replication at an accelerated rate (34). The saturation of the embryonic nuclei by a factor involved in DNA replication might enable this regulation. DNA replication takes place exclusively on the nuclear matrix during early development (34), and it has been observed that c-Myc can cofractionate with matrix-related structures in several cases (19, 20, 78). The exceptional rate of genomic replication during early development may require preformed structures, as found for the elements of the nuclear envelope (76). Both the size of the c-Myc complex (15S) and its rapid capture by the nuclei after fertilization are consistent with its association with such structures. After the midblastula transition, the amount of p64 c-Myc per genome becomes close to the value observed in a somatic cell in culture. At this time, transcription resumes in the embryo, the cell cycle increases in length, and G_1 and G_2 phases are reintroduced (55). This transition may therefore be important for the reorganization of the mode of genomic replication.

This model may also explain the apparent conflicting data concerning roles for c-Myc in both replication and gene expression. We suggest that during early development, when replication does not rely on specific sequences, c-Myc is involved in a functional complex adapted to such regulation. The absence of a c-Myc/Max complex able to specifically bind DNA during this period is also in favor of a role different from that

observed in somatic cells in culture. In addition, this view offers new insights into the oncogenic activity of c-Myc. The overexpression of c-Myc may cause cell immortalization by reintroducing a situation normally adapted only to early development.

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