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Stimulation of *Xenopus* oocyte protein synthesis by microinjected adenovirus RNA

(translational control/two-dimensional gel electrophoresis/hybridization selection/mRNA recruitment)

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ABSTRACT **The ability of injected heterologous mRNAs to compete with endogenous mRNAs in *Xenopus* oocytes was assayed. In confirmation of previous reports, globin mRNA translation in oocytes results in a concomitant decrease in endogenous protein synthesis. In contrast, injection of adenovirus 5 mRNA into the oocyte results in a stimulation of endogenous protein synthesis. The stimulation is dose dependent and does not require nuclear transcription in the oocyte. Preliminary mapping data suggest that the stimulatory RNA is a product of one of the viral immediate early genes.**

The *Xenopus* oocyte has been used as a test system to study transcription (1, 2), translation (3–6), and secretion (5, 6) of heterologous molecules after microinjection. Many different mRNAs from a variety of sources have been injected into the oocyte cytoplasm, and in most cases they were efficiently translated. The translation of these molecules was always accompanied by a corresponding decrease in endogenous protein synthesis such that the level of total synthesis in the oocyte remained relatively constant (4, 7, 8). These observations led to the conclusion that there is a competition between endogenous and injected mRNAs for a rate-limiting translational component.

We report in this communication that, in contrast to all other mRNAs tested, a poly(A)-RNA fraction isolated from adenovirus-5 infected HeLa cells stimulates oocyte protein synthesis after microinjection. The stimulation is dose dependent and does not require transcription. We have further mapped the stimulatory RNA to an immediate early gene of the viral genome. The accumulated data are discussed in relationship to the regulation of protein synthesis in oocytes and in adenovirus-infected HeLa cells.

MATERIALS AND METHODS

Collection and Culture of Oocytes. Female *Xenopus laevis* were obtained from South Africa (South African Snake Farms, Fish Hoek). Stage VI oocytes were selected as described (9) and cultured in OR-2 medium (10) or Eppig and Dumont medium (11). For some experiments, oocytes were manually enucleated by incubating them in Ca^{2+}-free OR-2 medium containing 0.15% collagenase (Sigma, type II) for 2–3 hr. This removes most of the follicle cells and promotes healing after enucleation. A hole was then made in the animal pole and the germinal vesicle was gently squeezed out. Enucleated oocytes were allowed to heal for 30–60 min before they were injected with RNA. Only those oocytes whose germinal vesicles were removed intact were used for subsequent experiments.

Growth and Purification of Virus. Wild-type adenovirus type 5 (Ad-5) (H5 300) was originally obtained from H. Ginsberg. It was grown and purified from suspension cultures of HeLa cells as described (12). H5*dl*312 (13) was grown on 293 (human) cells.

RNA Preparation. Total cytoplasmic RNA was prepared from suspension cultures of HeLa cells (14). Late RNA was prepared 20 hr after infection from cells infected at a multiplicity of 20–40 plaque-forming units/cell. Early RNA was prepared from cells infected at the same multiplicity at 16 hr after infection; cytosine arabinonucleoside (araC) was present (25 μg/ml) from 1 hr after infection to prevent DNA synthesis and entry into the late phase of infection. This treatment allows accumulation of early viral sequences. In those cases in which anisomycin was used, it was added to the cells at 100 μM 0.5 hr prior to infection and the cells were harvested 16 hr after infection. RNA was prepared from *dl*312-infected cells by following the protocol described above for the preparation of early RNA. In some cases, RNA was isolated from uninfected HeLa cells that were cultured in the presence or absence of araC. Globin mRNA was isolated from rabbit reticulocytes (15).

Poly(A)-containing RNAs were heat denatured (60°C) and selected by three passes over an oligo(dT)-cellulose column as described (15). The RNAs were precipitated with ethanol and suspended in sterile water at 1–2 mg/ml. They were injected into oocytes in volumes of 20–40 nl (15).

Measurement of Protein Synthesis. Absolute rates of protein synthesis in oocytes were determined as described (9, 16, 17). Briefly, oocytes were microinjected with 20 nl of [^{3}H]leucine (4 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), delivering 4.4×10^5 dpm and 50 pmol of leucine per oocyte. This concentration of leucine expands the endogenous leucine pool (50 ± 10 pmol) sufficiently to generate linear incorporation kinetics for 60–90 min. Rates of synthesis (ng of protein per oocyte per hr) then were calculated from the slopes of incorporation curves and the leucine specific activity, using an average leucine pool size of 100 pmol.

Polyacrylamide Gel Electrophoresis. Radiolabeled oocytes were homogenized in 50 mM NaCl and 0.5 mM phenylmethylsulfonylfluoride and the yolk was removed by centrifugation. The supernatant protein was precipitated in 4 vol of ethanol at −20°C, collected by centrifugation, and suspended in NaDodSO$_4$ sample buffer and electrophoresed in NaDodSO$_4$/12.5% acrylamide gels (18) or suspended in lysis buffer and electrophoresed in two dimensions according to O'Farrell (19). The second dimension consisted of 10% acrylamide. Gels were impregnated with 2,5-diphenyloxazole and fluorographed (20).

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Abbreviations: Ad-5, adenovirus type 5; araC, cytosine arabinonucleoside.

Hybridization Selection. Hybridization selection was performed by the basic procedure of Ricciardi *et al.* (21). Eighty micrograms of purified viral DNA was denatured and applied to nitrocellulose paper (BA 85, Schleicher & Schuell) in 1.5 M NaCl/0.15 M sodium citrate. The paper (1 cm^2) was air dried and baked at 80°C for 2 hr. Fifty micrograms of poly(A)-RNA from HeLa cells infected for 24 hr with wild-type virus was incubated with the filters for 2 hr at 50°C. The filters were washed and the RNA was eluted. Both the bound and unbound fractions were precipitated with ethanol, suspended in sterile water, and used for oocyte microinjection.

RESULTS

Laskey *et al.* (7) clearly demonstrated that the injection of heterologous mRNA into oocytes results in the synthesis of message-specific protein accompanied by a reciprocal decrease in the synthesis of endogenous protein. An example of this is shown qualitatively in Fig. 1*A*; globin mRNA was injected into oocytes at various concentrations and 12 hr later [^{3}H]leucine was injected. After 1 hr, oocyte proteins were isolated and separated by gel electrophoresis. As shown in Fig. 1*A*, the synthesis of globin increases as more globin mRNA is injected; however, the synthesis of endogenous proteins concomitantly decreases. In contrast to this result, the injection of mRNA from Ad-5-infected HeLa cells resulted in a marked overall increase in the synthesis of total proteins (Fig. 1*B*). The increase was proportional to the amount of mRNA injected. By one-dimensional gel electrophoresis, two viral mRNA-specific proteins are seen; they comigrate with the fiber protein of molecular weight 60,000 and protein IX of molecular weight 12,500. However, analysis of the same protein samples by two-dimensional gel electrophoresis reveals that many other nonoocyte proteins are synthesized, of both viral and HeLa cell origin (see below).

Adenovirus RNA Stimulates Endogenous Protein Synthesis. In order to determine if the increase after RNA injection is due to the synthesis of heterologous proteins only or is also due to a stimulation of oocyte protein synthesis, we have examined the relative synthesis of individual polypeptides from control and RNA-injected oocytes on two-dimensional gels. RNAs were isolated during the early and late phases of infection. The early RNA contains exclusively viral RNA from the early genes, whereas the late RNA, although predominantly containing RNA from the late genes, also contains some early sequences. This was done in an attempt to determine if the stimulatory RNA is present in greatest abundance at any particular time after viral infection of HeLa cells (see also Fig. 4). Fig. 2 shows the pattern of polypeptide synthesis from control oocytes (*A*) and early- (*B*) and late- (*C*) RNA-injected oocytes. Of the approximately 250 polypeptides represented in the control panel, it appears that almost all increased in synthesis after RNA injection. In addition, there were about 20 new polypeptides synthesized after RNA injection that are presumed to be either viral or HeLa cell proteins. Because it is possible that some oocyte polypeptides that apparently increased in synthesis after RNA injection might actually be a composite of oocyte and HeLa or viral proteins that have the same two-dimensional coordinates, we have translated the RNA *in vitro* and have analyzed the products on two-dimensional gels. Fig. 2*D* shows the pattern of labeled polypeptides that are packaged into the virion, and Fig. 2 *E* and *F* show the pattern of *in vitro* translation products of early and late RNAs. Although the early RNA pattern is quite complex, it is readily apparent that when this is compared to the early RNA pattern from injected oocytes (B), many polypeptides that increased in synthesis after RNA injection do not correspond with an *in vitro* translation product. This is even more obvious when the two-dimensional gel patterns of late-RNA-injected oocytes (Fig. 2*C*) and late RNA *in vitro* translation products (Fig. 2*F*) are compared. In late infected cells, the majority of the cytoplasmic message is virus specific, and this is reflected in the decreased complexity of *in vitro* translation products (Fig. 2*F*) compared to that obtained with early mRNA (Fig. 2*E*). In contrast, the complexity and intensity of those proteins clearly recognized as oocyte specific is comparable regardless of whether early or late mRNA is injected (Fig. 2 *B* and *C*). Thus, we conclude that the increase in protein synthesis in mRNA-injected oocytes is due to an actual stimulation of oocyte translation.

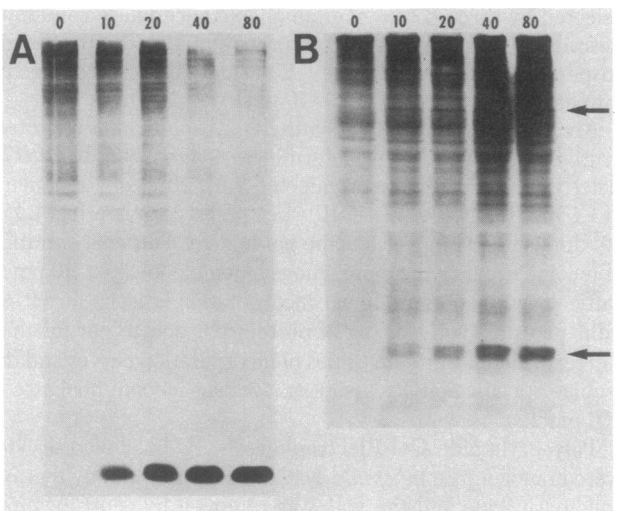

FIG. 1. $NaDodSO_4$ gel fluorograms of the proteins synthesized in mRNA-injected oocytes. Oocytes were injected with 0, 10, 20, 40, or 80 ng of globin mRNA (*A*) or late poly(A)-RNA from adenovirus-infected HeLa cells (*B*), cultured for 12–15 hr, and then injected with [^{3}H]leucine. After an additional 1 hr of culture, the oocytes were homogenized and the ethanol-precipitated protein was prepared for electrophoresis. The same amount of protein (from four oocytes) was loaded onto each lane. The arrows in *B* denote viral proteins (fiber, molecular weight 60,000, and protein IX, molecular weight 12,500) synthesized in oocytes after RNA injection. The gel in *A* has been presented previously (15).

To obtain more quantitative data, the absolute rates of protein synthesis from control and RNA-injected oocytes were determined. Fig. 3 shows an example of the incorporation of [^{3}H]leucine into protein by oocytes injected 15 hr previously with late mRNA from adenovirus-infected HeLa cells and by uninjected controls. The rate of incorporation for the mRNA-injected oocytes was 1.6-fold greater than that of the control. The rate for oocytes injected previously with mRNA from uninfected HeLa cells was almost identical to that for uninjected oocytes, and the rate for oocytes injected with early mRNA was virtually identical to that for late mRNA (data not included).

The absolute rates of protein synthesis in oocytes injected with several concentrations of early and late mRNA, as well as in the two controls, are shown in Fig. 4. It is apparent that as the concentration of either early or late mRNA increases, the degree of stimulation above control levels increases. The greatest difference was obtained at the highest concentration used. In these particular experiments, the rate of protein synthesis in oocytes injected with 80 ng of late mRNA from infected HeLa cells averaged 1.8-fold greater than in control oocytes injected with mRNA from uninfected HeLa cells.

The magnitude of the response of oocytes to injected viral mRNA has been quite variable, as indicated in Fig. 4. For example, we have performed 21 separate experiments in which oocytes from several different females were injected with late mRNA and compared with controls. In 20 of the cases, the level

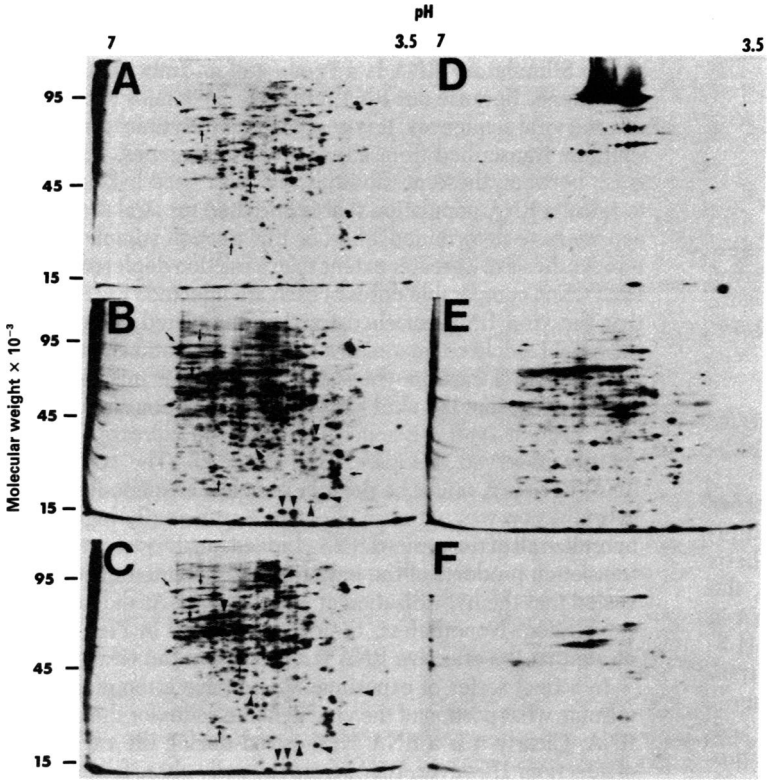

FIG. 2. Two-dimensional gel electrophoresis patterns of polypeptides synthesized in mRNA-injected oocytes or in a rabbit reticulocyte lysate. Noninjected controls (*A*) or oocytes injected with 80 ng of early (*B*) or late (*C*) RNA were cultured for 16 hr, injected with [^{35}S]methionine (about 200,000 cpm), and cultured for an additional hour. The oocytes were then homogenized and the same amount of protein (from five oocytes) was applied to the first-dimension isoelectric focusing gel. There was about twice as much trichloroacetic acid-precipitable radioactivity from the early- and late-RNA-injected oocytes compared to controls. The patterns of polypeptide synthesis from [^{35}S]methionine labeled capsid polypeptides (*D*) and the *in vitro* translational products of early (*E*) and late (*F*) RNAs are shown. The arrows denote just a few oocyte polypeptides that increased in synthesis after injection of early or late RNA and that did not have the same two-dimensional coordinates as polypeptides synthesized *in vitro*. The arrowheads denote polypeptides synthesized in oocytes only after RNA injection. Each experiment was performed three times.

of incorporation in oocytes injected with the viral mRNA preparation was greater than in control (noninjected) oocytes. In more than 80% of the experiments, the degree of stimulation ranged between 1.3- and 2.2-fold. The mean value (±SD) for these experiments was 1.55 ± 0.24 and for all experiments was 1.46 ± 0.29.

Variability has been observed frequently in studies concerned with quantitative measurements, especially when oocytes from different females were compared. For example, the well-documented increase in protein synthetic rate in oocytes induced to mature with progesterone varies between 1.3- and 2.9-fold, depending on the reproductive condition of individual females (9). Asselbergs *et al.* (8) further have reported that oocytes from different females vary considerably in their ability to translate injected messages. On the other hand, it is important to emphasize that in the present study we observed no stimulation in total protein synthesis when mRNA from uninfected HeLa cells was injected into oocytes (Fig. 4). Similarly, injection of several other messages has not resulted in any increase in total

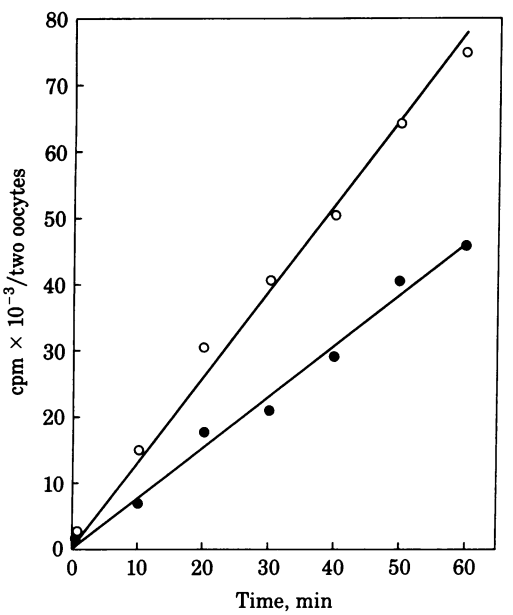

FIG. 3. Kinetics of [^{3}H]leucine incorporation in RNA-injected oocytes. Oocytes were injected with 80 ng of late viral mRNA and cultured for 15 hr. After injection of [^{3}H]leucine (0.4 μCi per oocyte), the hot perchloric acid-insoluble radioactive material was determined at several subsequent times. ●, Incorporation for noninjected controls; ○, incorporation for RNA-injected oocytes.

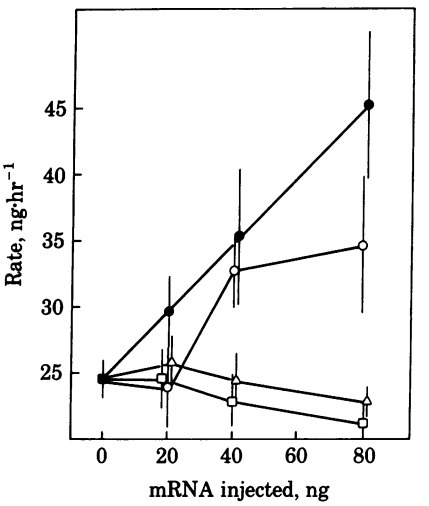

FIG. 4. Absolute rates of protein synthesis of RNA-injected oocytes. Oocytes were injected with various amounts of late (●) or early (○) RNA or with RNA from uninfected HeLa cells cultured in the absence (△) or presence (□) of araC. The rate from noninjected control oocytes is shown (■). Vertical bars refer to standard deviations determined from at least three replicates. Rates were determined 15–18 hr after RNA injection.

Table 1. Absolute rates of protein synthesis of normal and enucleated mRNA-injected oocytes

Oocytes	Nucleus	Rate, ng·hr^{-1} Mean	Range
Control	Present	14.6	13.3–15.9
mRNA-injected	Present	19.2	18.8–19.6
Control	Absent	13.3	10.0–16.3
mRNA-injected	Absent	20.0	17.5–22.5

The rates are means of two determinations, each from a different animal. Oocytes were enucleated and allowed to heal for 1 hr. Eighty nanograms of RNA was then injected and the kinetics of protein synthesis were determined 16 hr later.

oocyte protein synthesis (4, 7). We conclude, therefore, that any stimulation resulting from the injection of mRNA from Ad-5-infected HeLa cells is significant.

Protein Synthesis in the Absence of Transcription. In order to determine if transcription is required for the observed increase in synthesis, we measured the rates of protein synthesis in normal and enucleated oocytes. As shown in Table 1, the stimulation of protein synthesis by 80 ng of adenovirus RNA in control and enucleated oocytes was essentially the same (1.3- to 1.5-fold). Thus, the oocyte nucleus, and hence nuclear transcription, is not required for the protein synthesis increase.

Functional Stability of Injected RNA. Several studies suggest that after injected mRNA associates with polysomes or protein factors, it becomes stable at least for several days (4, 22, 23). If the RNA that stimulates oocyte protein synthesis is stable after injection and continues to be translated, then the protein synthesis rates might be expected to continue to increase with time. To determine if the stimulation can be increased by simply prolonging the culture time after injection, protein synthesis rates were measured at several times after RNA injection (Fig. 5). Surprisingly, the rate of protein synthesis continued to increase only up to about 18 hr after injection of the message preparation; then it began to decline and was almost equal to the control rate 36 hours after injection. A second injection at a time when the rate was decreasing but still high maintained the high synthesis rate for about 24 hours; after that time, the rate again began to decline and approached the control level of synthesis (Fig. 5). In other experiments, a second injection of RNA at 36 hr, when the synthesis rate from previously injected oocytes was at the control level, stimulated a second increase in protein synthesis, albeit not to the same magnitude observed after the first injection (data not shown).

The Stimulatory RNA Is a Product of an Immediate Early Viral Gene. Because our RNA preparation contains both HeLa cell and viral sequences, it is possible that the stimulatory RNA could be transcribed from either of those genomes. To distinguish between these possibilities, we have used hybridization to select a RNA population that is enriched for viral sequences and we have determined whether this fraction stimulates protein synthesis to a greater extent than a fraction depleted in viral RNA when comparable doses of each are injected. Fig. 6 shows that the viral RNA-enriched fraction stimulated protein synthesis by 1.9-fold compared to a 1.4-fold stimulation by the viral RNA-depleted fraction when 80 ng of each was injected. One might expect that the viral RNA-depleted fraction should stimulate protein synthesis much less than the increase that was actually observed (compare Figs. 4 and 6). The stimulatory RNA, however, might be present in so low an amount that our hybridization was not carried out to a sufficiently high C_0t to hybridize all of the desired RNA. Indeed, analysis of the *in vitro* translation products of the enriched and depleted fractions revealed that the hybridization of some viral RNAs did not get to completion. Nonetheless, the data presented in Fig. 6 do indicate that the effective RNA is a product of the viral genome.

In a final series of experiments, we have attempted to determine what portion of the viral genome codes for the effective RNA. Clearly it is a RNA synthesized during the early phase of infection. However, RNAs from the early phase fall into three specific classes: the immediate early, the pre-early, and the delayed early (24). The immediate early genes are expressed in the absence of any protein synthesis. The pre-early phase involves the expression of region E1a which, in turn, is necessary for the expression of the delayed early genes. The viral mutant *dl*312 has undergone a deletion that removes almost the entire E1a region (13); as a result the delayed early genes are not expressed, but the immediate early genes should be unaffected (14, 24). Poly(A)-RNA was isolated from *dl*312-infected HeLa cells cultured in the presence of araC and from wild-type virus-infected cells cultured in the presence of 100 μM anisomycin, a potent inhibitor of protein synthesis. Under such conditions, only immediate early genes are expressed (24). Eighty nanograms of these RNAs, as well as late RNA, was injected into oocytes and the protein synthesis rates were determined. The rates were 21.8 ng/hr for controls and 34.7 ng/hr (*dl*312), 30.6

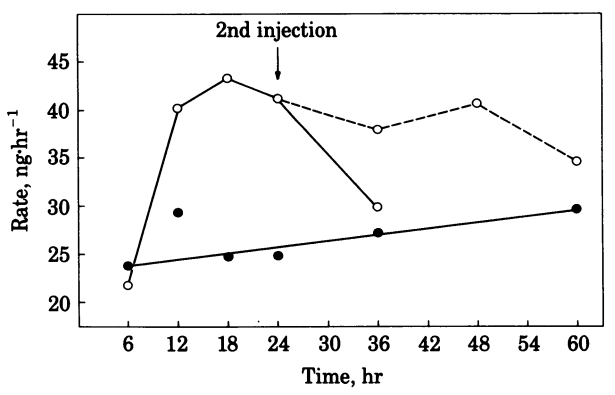

FIG. 5. Stimulation of protein synthesis as a function of time in RNA-injected oocytes. At several times after injection, rates of protein synthesis were determined as described in Fig. 2 for control oocytes (●) and oocytes injected with 80 ng of late RNA (○——○). One group of oocytes received a second injection of 80 ng of late RNA 24 hr after the first injection (○– – –○).

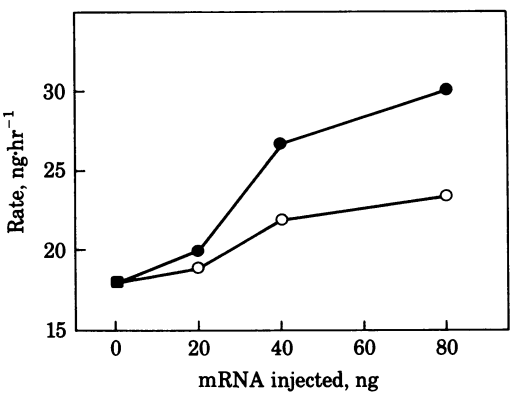

FIG. 6. Rates of protein synthesis in oocytes injected with RNA selected by hybridization. Total viral DNA bound to nitrocellulose filters was hybridized to total late RNA. After washing and elution, the RNAs in the bound (●) and unbound (○) fractions were precipitated with ethanol and injected into oocytes. The rates of protein synthesis were determined 15 hr later. The values shown are the means from two experiments.

ng/hr (anisomycin), and 35.0 ng/hr (late) for the three experimental groups. In two additional experiments, the stimulation of protein synthesis from oocytes injected with 80 ng of RNA from *dl*312-infected HeLa cells was 1.4- and 1.5-fold. Therefore, the above data strongly suggest that the stimulatory RNA is a product of one of the immediate early genes.

DISCUSSION

The induction of oocyte maturation, by either steroid hormones or the proteinaceous maturation-promoting factor results in an approximate doubling of the rate of protein synthesis (9). Recent studies have shown that this increase does not result from changes in translational efficiency (15). Thus, the data indicated that increased protein synthesis during oocyte maturation requires a recruitment of mRNA. However, although the full-grown *Xenopus* oocyte translates only a few percent of the mRNA mass thought to be present by the end of oogenesis, it now seems certain that message availability alone does not limit protein synthesis. This has been shown most clearly by the demonstration that injected messages usually compete with endogenous mRNA for a limited translational capacity in the oocyte (4, 7, 8). The nature of the limiting component(s) is not known, but presumably the components include specific proteins.

In this communication, we demonstrate that the injection of adenovirus RNA stimulates endogenous protein synthesis in recipient oocytes. The stimulation appears to be similar to that observed after treatment of oocytes with progesterone or maturation-promoting factor in that the increased synthetic rate does not result from a change in translational efficiency (data not shown) as defined previously (15). Thus, as in the situation described above, the adenovirus message preparation would result both in message recruitment and a change in the availability of whatever other component(s) limits translation.

The actual molecular events involved in the stimulatory effects of the adenovirus RNA remain unknown. However, maximal stimulation of oocyte protein synthesis occurs about 18 hr after injection of the RNA preparation. This is at least 12 hr beyond the time when injected message would have been expected to reach its maximal rate of translation (4). This suggests that stimulation is due not to the RNA itself but to a translational product that must be accumulated to a certain level. The additional observation that the increased synthesis rate is not maintained beyond 18 hr would suggest further that the translational product is unstable and that the template either turns over also or ceases to be translated beyond a certain time. Either possibility implies translational regulation of the adenovirus message. Evidence for the translational regulation of endogenous (25) and injected mRNAs (4) has been presented previously.

The stimulatory RNA appears to be a product of an immediate early viral gene. These are defined as genes that are expressed in the absence of any protein synthesis and are independent of regulation by the pre-early E1a product. Two such genes have been described. One lies in the region between 30 and 39 map units on the viral genome and contains the tripartite leader sequence that is found on most of the late viral messages (24); messages selected with DNA from this region translated *in vitro* give two closely related polypeptides of molecular weights 52,000 and 55,000. The other lies between 17 and 21.5 map units and encodes a product of molecular weight 13,500. All the immediate early products are found in very low amounts at early times of infection but are more abundant at late times. This could explain why late RNA consistently gave the best stimulation in injected oocytes (cf. Fig. 4). From the results presented here, we cannot distinguish which of the immediate early products is involved in the stimulation; additional experiments using specific DNA-selected mRNA species should provide the answer.

It is not known whether an immediate early gene product stimulates total protein synthesis in infected HeLa cells. However, we have preliminary data to suggest that it does. A transient increase in total protein synthesis (by about 1.5-fold) is observed early after infection of HeLa cells with either wild-type virus or with *dl*312 (unpublished observation). In addition, the increases in protein synthesis in oocytes after RNA injection or in HeLa cells after viral infection occur at similar times if the effect of incubation temperature (2.7-fold increase for 10°C increase) is taken into account. Because of these similarities, we suggest that the *Xenopus* oocyte might also be a useful system to study the mechanism of viral infection, especially at the translational level.

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