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DNA-ligase activity in axolotl early development: evidence for a multilevel regulation of gene expression

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INTRODUCTION

DNA-ligase activity in eukaryotic cells is carried out by two different molecular forms of the enzyme. This molecular duality, first described in mammals (Söderhäll & Lindhal, 1973), has been reported in chicken (David, 1977), amphibians (Carré, Signoret, Lefresne & David, 1981), fish, sea urchin and crab (Signoret & David, 1986). Depending on the developmental stage and the tissue considered, types of cell are available that express exclusively either one or the other of the two DNA-ligases with a defined level of activity. DNA-ligase I and DNA-ligase II, encoded by distinct structural genes (Thiebaud *et al.* 1985), can be unambiguously characterized and the advantages offered by the early amphibian embryo have made possible the identification of some basic features of their regulation.

THE GENE FOR DNA-LIGASE I EXISTS IN TWO REGULATED STATES

The heavy molecular form of the enzyme, referred to as DNA-ligase I, is generally present in proliferating tissues. In the axolotl, DNA-ligase I (8S) is specific for embryonic stages. It cannot be detected in the unfertilized egg, sperm or differentiated tissues such as liver or muscle. Following fertilization or artificial activation, the gene for DNA-ligase I of the female pronucleus undergoes the complete process of expression, i.e. transcription and translation, resulting in the characteristic biologically active product (Signoret, David, Lefresne & Houillon, 1983; Lefresne, David & Signoret, 1983). However, this expression depends on a preliminary DNA replication that occurs normally between 2½ h and 3½ h following activation at 18 °C. Thus, the gene for DNA-ligase I is overtly expressed at the 1-cell stage. In fact, the normal level of activity for the heavy molecular form of the enzyme has been established by the time the egg enters cleavage, 7 h after

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activation. This situation is representative of the unrepressed and fully expressed state of the gene. In the same cytoplasmic environment, and following egg activation and DNA replication, the gene for DNA-ligase I introduced by the male pronucleus is unable to direct any production of the corresponding enzyme. Haploid androgenetic axolotl embryos develop devoid of the 8S DNA-ligase characteristic of control embryos (Fig. 1A,B). The paternal gene for DNA-ligase I is in a repressed state.

Nuclear transfer offers the possibility of testing the capacities of selected nuclei after implantation into the egg cytoplasm. Individual nuclei taken from a blastula or gastrula are able to replace the female pronucleus in the expression of the gene for DNA-ligase I. This gene is assumed to exist in the unrepressed state in any nucleus from early embryonic stages. However, DNA replication in the egg cytoplasm is a prerequisite for the expression of the gene for implanted nuclei as well as for the original female pronucleus.

When the donor embryo is a haploid androgenetic blastula or gastrula, there is no production of DNA-ligase I in the host cytoplasm, and the nuclei from androgenetic embryos appear to carry the corresponding gene in the repressed state. Consequently, the regulated states of the gene for DNA-ligase I in the axolotl, either repressed or unrepressed, exhibit a significant degree of stability following nuclear transplantation, and appear to be selfpropagating through cell multiplication.

Two homologous genomes, differentially regulated and exposed to the same cytoplasmic influence, are able in this particular case to retain their respective aptitudes (or inaptitudes). Moreover, the regulated state of this gene can be transmitted differentially for paternal and maternal alleles from the germ cells to the zygotic nucleus of the next generation. This situation could conceivably concern other genes in the same way. If such is the case, paternal and maternal participation in fertilization differ in this respect and normal early gene expression would require both male and female participants of the regulated genome. This consideration could account for the necessity of the two types of pronuclei for normal development, recently established on an experimental basis (Surani, 1985).

The stability of the regulated state of the gene for DNA-ligase I following nuclear transfer in the axolotl egg has been confirmed for a variety of donor cells taken from foreign species, notably *Pleurodeles*. When produced, the enzyme shows the molecular characteristics and immunological specificity of the implanted material, clearly distinct from the host counterpart (Fig. 1C,D).

Fig. 1. Evidence for stable regulated states of the gene for DNA-ligase I. Left, diagram of the experiment; centre, DNA-ligase activity after sucrose gradient centrifugation; right, peaks of activity characterized. (A) Unfertilized egg and diploid blastula 24 h, (B) haploid androgenetic blastula 24 h, (C) axolotl activated egg implanted with *Pleurodeles* blastula nucleus, diploid, (D) axolotl activated egg, implanted with *Pleurodeles* haploid androgenetic nucleus, (E) same experiment as D, except a transient treatment with spermine of the transplanted nucleus. Material and methods are reviewed in Signoret & David, 1986.

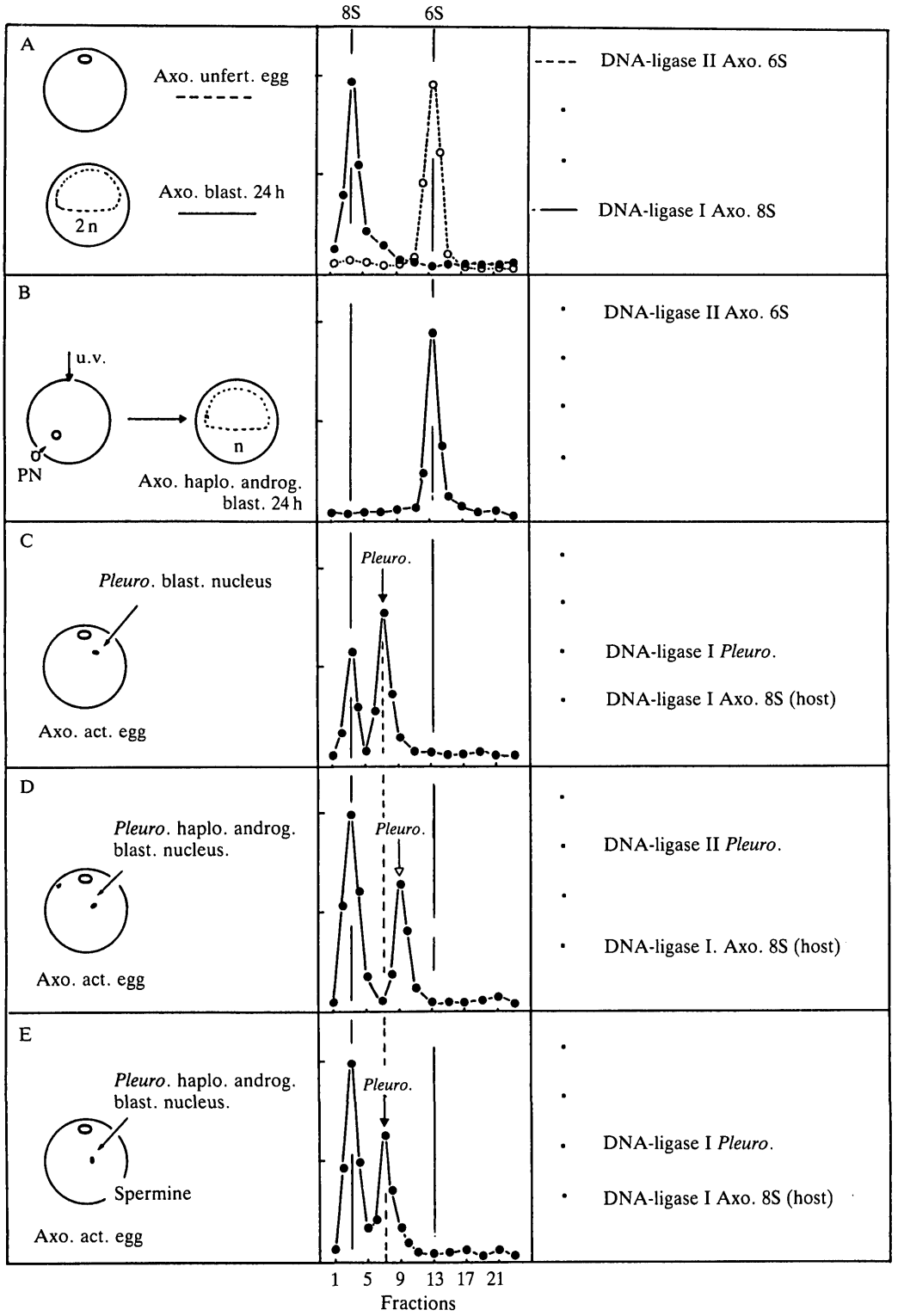
8S
6S
A
Axo. unfert. egg
Axo. blast. 24 h
2n
DNA-ligase II Axo. 6S
DNA-ligase I Axo. 8S
B
u.v.
PN
n
Axo. haplo. androg. blast. 24 h
DNA-ligase II Axo. 6S
C
Pleuro. blast. nucleus
Axo. act. egg
Pleuro.
DNA-ligase I *Pleuro.*
DNA-ligase I Axo. 8S (host)
D
Pleuro. haplo. androg. blast. nucleus.
Axo. act. egg
Pleuro.
DNA-ligase II *Pleuro.*
DNA-ligase I. Axo. 8S (host)
E
Pleuro. haplo. androg. blast. nucleus.
Spermine
Axo. act. egg
Pleuro.
DNA-ligase I *Pleuro.*
DNA-ligase I Axo. 8S (host)
1 5 9 13 17 21
Fractions

Fig. 1

The repressed state of the gene for DNA-ligase I, although significantly stable and clearly autoreproducible, is far from irreversible. Transitory treatments of the donor material have been devised that induce a reprogramming of the gene. When nuclei taken from cells devoid of DNA-ligase I are treated with 0·35 M-NaCl and then implanted in the egg cytoplasm, production of DNA-ligase I can be detected, revealing some extent of gene derepression. Treatment with spermine is still more effective; genes for DNA-ligase I are entirely switched to the unrepressed state (Fig. 1E). Spermine and NaCl are thought to encourage the mobility of basic nucleoproteins, and hence possibly to alter chromatin structure. We suggest that the DNA with its cooperatively bound histones constitute a selfpropagating structure resulting in the local inaccessibility of genes (repressed state). This structure could be modified by experimental treatments or biological processes (unrepressed state). Then, during particular events such as DNA replication, transcription factors may gain access to the DNA and constitute an active initiation complex (expression). A mechanism of this type has been proposed by Weintraub (1985). Starting from the repressed state, gene expression would involve two distinct steps: a structural change, which could occur some time during egg maturation, and transcriptional activity acquired with DNA replication. The former is maintained after cessation of the causative stimulus, and the 'open' structure is autoreproducible. The latter is periodically reset at every round of DNA replication. There is no evidence for any species specificity in this mechanism. It operates normally for a nucleus of foreign origin in a given cytoplasm.

THE 'EXCLUSION PROCESS', AN INTERRELATION BETWEEN DNA-LIGASE I AND DNA-LIGASE II

We have just considered in the preceding section the regulation of DNA-ligase I production, the heavy molecular form of the enzyme. Extensive observations in the axolotl and other material have established that in cells possessing this DNA-ligase the alternative isoenzyme, DNA-ligase II, is generally undetectable. Reciprocally, cells devoid of DNA-ligase I do possess the light molecular form of the enzyme, DNA-ligase II. It is noteworthy that the two molecular entities depend on distinct structural genes, and that any possible conversion from one to the other has been definitely ruled out (Thiebaud *et al.* 1985). Never has any cell type been found that produces both enzymes at once. The occasional coexistence of DNA-ligase I and II is a transient situation related to a shift from one type to the other. DNA-ligase II (6S) is present in the unfertilized axolotl egg and in sperm, disappearing after egg activation. The light molecular form is undetectable during embryonic stages of development when DNA-ligase I (8S) accounts for all of the DNA-ligase activity. In haploid androgenetic embryos DNA-ligase II is maintained at its normal level, and no DNA-ligase I is produced. Experimental evidence has led to the proposition that the gene for DNA-ligase II exists in only one state of regulation, namely expressible, although not necessarily expressed. Its expression depends on whether or not the gene for DNA-ligase I is expressed.

According to this scheme the female genome possesses the unrepressed gene for DNA-ligase I, and the expression of this gene inhibits any expression of the maternal gene for DNA-ligase II. The same control is exerted over the male set of genes. The paternal copy of the gene for DNA-ligase I (8S) is repressed, but the paternal gene for DNA-ligase II in the zygotic genome is inhibited by the expression of the maternally inherited allele for DNA-ligase I. A diploid embryonic cell is assumed to possess two genes for DNA-ligase I, one being repressed while the other is unrepressed and actively expressed; and two genes for DNA-ligase II, both potentially expressible but inhibited by the expression of the ligase I gene (Fig. 2A,B,C).

The process can be further explored in systems resulting from nuclear transplantation. It appears as a rule that the inhibiting signal delivered by a single gene for DNA-ligase I is able to control all copies of the gene for DNA-ligase II lying in the same cytoplasm even when they are in other nuclei (Fig. 2D). This remarkable interrelation has been described as the 'exclusion process', and deserves some more consideration.

A very provocative result comes out from interspecific nucleocytoplasmic combinations, such as those involving *Pleurodeles* and axolotl (Signoret *et al.* 1983). The exclusion process appears strictly species specific and fails to operate between species. In the same cytoplasm simultaneous expression of the axolotl gene for DNA-ligase I and *Pleurodeles* gene for DNA-ligase II is constantly observed (and *vice versa*). This means that an axolotl egg fertilized by *Pleurodeles* spermatozoa expresses axolotl DNA-ligase I and *Pleurodeles* DNA-ligase II. Also the nucleus of an androgenetic haploid embryonic cell of *Pleurodeles*, implanted in an activated axolotl egg, produces *Pleurodeles* DNA-ligase II, despite the endogenous expression of axolotl DNA-ligase I (Fig. 1D). Reciprocal combinations provide identical results. The inhibiting signal delivered by an active gene for DNA-ligase I controls genes for DNA-ligase II from its own species and lets the gene(s) for DNA-ligase II of foreign species be freely expressed.

The mechanism underlying species-specific exclusion is not clearly understood, but interesting indications have come out from nuclear transplantations associated with antimetabolites. Nuclei from advanced gastrulae transplanted into egg cytoplasm produce DNA-ligase I. This synthesis results from complete gene expression, and is sensitive to transcription inhibitors. Unexpectedly, implanted nuclei treated with α-amanitin which cease to undergo *de novo* transcription due to the drug start to produce DNA-ligase II. A somewhat similar phenomenon has been reported by Tomkins, Levison, Boster & Dethlefson (1972) for tyrosine aminotransferase induced by actinomycin D. The interpretation proposed by Tomkins could apply to our system. We assume that in cells producing DNA-ligase I the gene for DNA-ligase II is transcribed, and the corresponding transcript accumulates in a stable form. Some particular product of the gene for DNA-ligase I, or of a gene coexpressed, associates with the transcripts for DNA-ligase II, and renders them unavailable for processing or recruitment. This factor is rather unstable and must be constantly renewed in order to ensure permanent

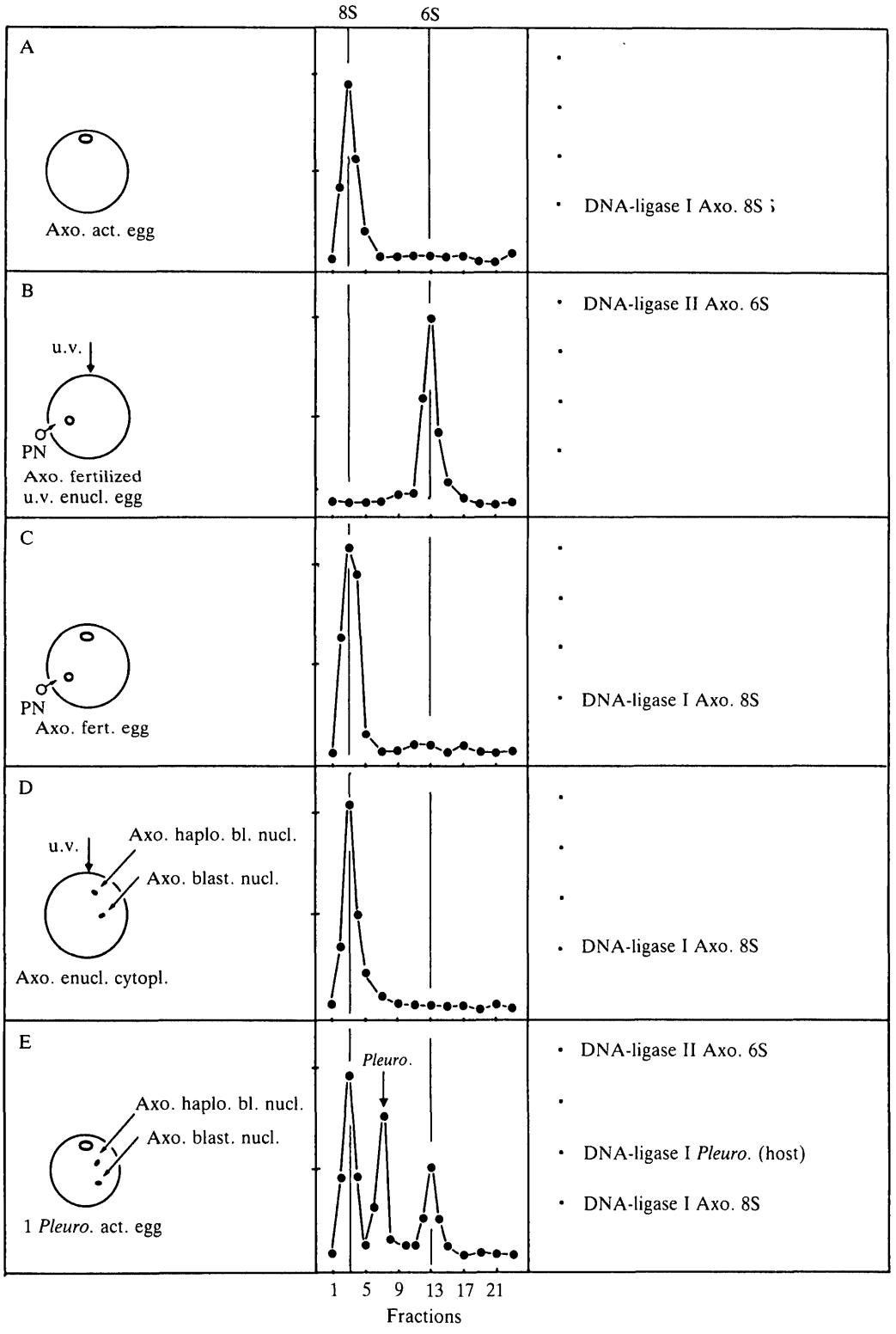

Fig. 2. 'Exclusion process' between DNA-ligase I and DNA-ligase II. Left, diagram of the experiment; centre, DNA-ligase activity after sucrose gradient centrifugation; right, peaks of activity characterized. (A) Female pronucleus, 9 h, (B) male pronucleus, 9 h, (C) zygotic nucleus, 9 h, (D) diploid blastula nucleus and haploid blastula nucleus, 9 h, (E) same experiment as D, except that the cytoplasm pertains to a foreign species. Material and methods are reviewed in Signoret & David, 1986.

inhibition of expression of the gene for DNA-ligase II. When transcription of the factor ceases in the presence of the drug, the inhibition is relieved and post-transcriptional expression of the gene can take place. According to this interpretation, the exclusion process involves a post-transcriptional negative control. If such a mechanism applies to other categories of genes, it could account for the control of batteries of genes by a few specific regulatory genes, and for differential and sequential gene expression when corresponding regulatory genes are turned off.

Species specificity of the process could conceivably be based upon a precise recognition between some inhibiting factor and the transcript to be masked. We have reported that the control is not limited to a single genome, but works in a trans-acting fashion on the associated genome of the same nucleus, or even on the set of genes of other nuclei, across the cytoplasm. However, a stringent species specificity appears in the transmission of the signal. The inhibition, typically exerted when nuclei are lying in their own cytoplasm, fails to work when the same nuclei are in the cytoplasm of foreign species (Fig. 2E).

The exclusion process operates upstream of the messenger RNAs and their translation, since, when RNAs coding, respectively, for DNA-ligase I and DNA-ligase II are injected in the same egg cytoplasm, both enzymic forms are produced at once, without any detectable interference.

DNA-LIGASE ACTIVITY IS CONTROLLED BY A QUANTITATIVE REGULATION

The level of DNA-ligase activity in the axolotl egg, measured per unit amount of protein, is remarkably constant. The steady state is established before the egg enters cleavage (7 h postactivation) and remains unmodified through the blastula and gastrula stages. There is no increase in DNA-ligase activity when the cytoplasm is endowed with an increasing number of nuclei, although any one of the thousands of nuclei of the embryo is able to express the normal level of enzymic activity. Similarly when the egg cytoplasm is implanted with 1, 2, 5 or 10 nuclei, there is never any dose effect, and the same standard level of DNA-ligase activity is constantly observed (Fig. 3A).

When RNA coding for either of the DNA-ligases is injected into the egg cytoplasm, the enzymic activity reaches, in a few hours, the typical plateau observed in control embryos. Injection of 10 times more RNA does not produce any significant increase in the level of DNA-ligase activity (Fig. 3B). The possibility of saturation of the translational equipment of the egg is very unlikely because the amount of mRNA injected is two orders of magnitude below the saturation dose, as determined for the *Xenopus* egg (Moar, Gurdon & Lane, 1971). Moreover, when axolotl RNA is injected into a *Pleurodeles*-activated egg there is no competition between synthesis of endogenous and exogenous forms of DNA-ligase I produced in the egg.

These observations, considered altogether, establish the existence of a delicate quantitative control of DNA-ligase activity in early development. The regulation,

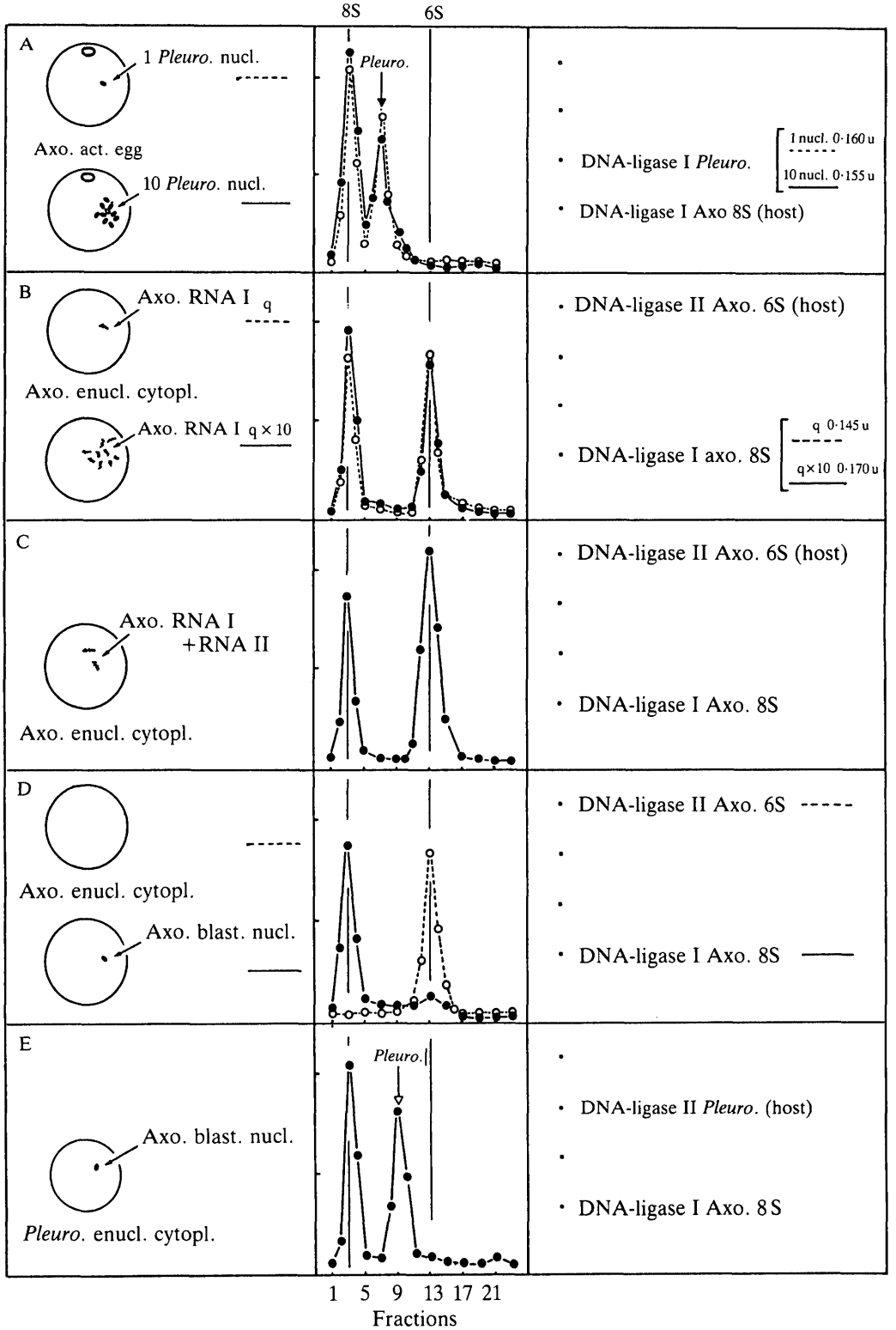
8S
6S
A
1 Pleuro. nucl.
Axo. act. egg
10 Pleuro. nucl.
Pleuro.
DNA-ligase I Pleuro.
1 nucl. 0·160 u
10 nucl. 0·155 u
DNA-ligase I Axo 8S (host)
B
Axo. RNA I q
Axo. enucl. cytopl.
Axo. RNA I q × 10
DNA-ligase II Axo. 6S (host)
DNA-ligase I axo. 8S
q 0·145 u
q×10 0·170 u
C
Axo. RNA I
+RNA II
Axo. enucl. cytopl.
DNA-ligase II Axo. 6S (host)
DNA-ligase I Axo. 8S
D
Axo. enucl. cytopl.
Axo. blast. nucl.
DNA-ligase II Axo. 6S
DNA-ligase I Axo. 8S
E
Axo. blast. nucl.
Pleuro. enucl. cytopl.
Pleuro.
DNA-ligase II Pleuro. (host)
DNA-ligase I Axo. 8S
1 5 9 13 17 21
Fractions

Fig. 3

probably based on a feed-back mechanism operates at a post-transcriptional level, independently of the available amount of the corresponding messenger RNA. Nevertheless the occurrence of such a process does not exclude a possible additional control operating upstream that could regulate the endogenous amount of mRNA by the rate of synthesis and degradation.

Experiments using interspecific nuclear transplantation or RNA injections show that the normal level is reached and maintained for any given form of DNA-ligase independently of the other molecular form(s) present (Fig. 3C). The quantitative control is carried out by each enzymic form of DNA-ligase on its own production, with a rigorous species specificity.

RELATIVE STABILITY AND SELECTIVE INHIBITION OF DNA-LIGASE II

DNA-ligase II (6S) in the axolotl egg appears to be a fairly stable enzyme. Living material treated with cycloheximide can be maintained for 24 h without any significant decrease in DNA-ligase II activity, even though protein synthesis is entirely abolished.

When a gene coding for axolotl DNA-ligase I undergoes expression in the egg cytoplasm, the activity of DNA-ligase II decreases dramatically within 3 h, and completely disappears a few hours later. The phenomenon occurs equally well under the control of the female pronucleus, following egg activation, or in the presence of an introduced nucleus resulting from nuclear transfer (Fig. 3D).

The striking replacement of one entity by the other could have suggested an erroneous interpretation, based upon a possible epigenetic modification of the enzyme, the heavy form of DNA-ligase being derived from the light one. Independent corroborative experiments have thoroughly ruled out this hypothesis. The two molecular forms of DNA-ligase are produced from distinct structural genes, and the expression of the gene for DNA-ligase I results in the specific quenching of DNA-ligase II activity. A convincing argument comes from interspecies combinations. When the gene for axolotl DNA-ligase I undergoes expression in enucleated *Pleurodeles* cytoplasm, the host DNA-ligase II remains undiminished, and the appearance of axolotl DNA-ligase I does not interfere with the endogenous enzyme (Fig. 3E). In this case the two molecular forms are clearly distinct, and it is reasonable to assume that such is the case when they pertain to the same species, and do not coexist.

This interrelation points to species-specific negative control exerted by a gene product on the terminal product of another nonallelic gene. This control could be

Fig. 3. Quantitative regulation and selective inhibition. Left, diagram of the experiment; centre, DNA-ligase activity after sucrose gradient centrifugation; right, peaks of activity characterized. (A) Implantation of 1 nucleus and of 10 nuclei, (B) injection of RNA, and 10 times more RNA, (C) injection of RNA coding for DNA-ligase I *and* RNA coding for DNA-ligase II, (D) disappearance of preexisting DNA-ligase II in enucleated cytoplasm injected with a blastula nucleus, 9 h, (E) persistence of DNA-ligase II in enucleated cytoplasm injected with a blastula nucleus of foreign species, 9 h. Material and methods are reviewed in Signoret & David, 1986.

achieved either by selective degradation of DNA-ligase II, or by the mediation of some specific inhibitor released in the cytoplasm.

CONCLUDING REMARKS

The molecular forms and enzymic activities of DNA-ligase in the axolotl egg are described in the present report as an original model for the study of the control of gene expression. Among other systems fruitfully investigated in eukaryotes, some involve genes so actively expressed in a specialized category of cell that they appear as prevalent. There is generally no question of overproduction for such genes, even when superprevalence ends in the death of the cell. Investigating the way a cell synthesizes a major protein furnishes precious information about gene expression, as compared to nonexpression, but is not likely to permit the exploration of subtle regulative mechanisms. Conversely, DNA-ligase plays a critical role in the proper economy of all living and selfreproducing cells. Far from the unselfish piling up of substances for the benefit of the whole organism, DNA-ligase synthesis is managed by the cell for its own use. Gene expression for a domestic enzyme must be submitted to regulation much more sophisticated and probably complex than genes for exported proteins or suicide accumulation. The cell has to adjust the end product activity very rapidly to quickly changing needs, and to guard against being overwhelmed by the active product.

Accordingly, we have presented evidence for an impressive cascade of controls, both qualitative and quantitative, and proposed interpretative mechanisms: cis-acting regulation of chromatin conformation, trans-acting effects of biological events, end-product feed-back regulation and modification of the entry exit balance. Moreover, interrelations between nonallelic genes introduce a non-reciprocal interdependence in their expression, operating in the nucleus at a post-transcriptional level, and in the cytoplasm, at the level of the terminal products.

Three concluding remarks appear of general importance: first, a multilevel system of regulation controls DNA-ligase activity, and probably shares common features with the processes controlling other enzymic activities in eukaryotes. The complexity of the whole mechanism accounts for the apparent paradox of long-lived gene products and short-term adjustment to the cell demand. Second, negative controls play a critical role in this series, and appear to be numerous and diversified, facing a few major positive regulations. Third, most of the process exhibits a stringent species specificity, probably relevant to precise molecular matching involved in the recognition of partner factors.

Further studies, combining molecular and experimental approaches, and extended to other favourable materials, will probably cast some light on the major steps of DNA-ligase activity regulation. The interpretation will greatly benefit from the current progress in gene regulation research in other systems, and will possibly contribute to the general understanding of the problem.

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