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GTP STIMULATES FUSION BETWEEN HOMOLOGOUS AND HETEROLOGOUS NUCLEAR MEMBRANES

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The tissue and species specificity of GTP-stimulated nuclear membrane fusion has been examined. The fusion capacity of the membranes of nuclei isolated from two different tissue sources and three different animal species was determined. In all cases the incubation of isolated nuclei in the presence of 0.5 mM GTP led to the pairing of nuclei and formation of continuous outer membranes between the nuclei as a result of membrane fusion. Experiments using mixtures of nuclei from the different sources demonstrated that hybrid nuclear membranes could be formed as a result of the fusion between the outer membranes of heterologous nuclear pairs. The results suggest that the capacity for nuclear membranes to fuse in the presence of GTP is highly conserved when viewed on an evolutionary basis.

Introduction

Previous in vitro studies have shown that physiological concentrations of the nucleotide guanosine triphosphate (GTP) can stimulate fusion between outer nuclear membranes [1], rough endoplasmic reticulum membranes [2,3] as well as between both these types of membranes [4]. The membrane specificity of GTP-stimulated fusion was indicated by the fact that GTP would not stimulate fusion between mitochondrial membranes or between Golgi membranes [4]. Because physiological concentrations of GTP can stimulate fusion between membranes of nuclear envelope and endoplasmic reticulum origin in vitro it was proposed that this nucleotide may play a role in nuclear envelope assembly in post-mitotic cells [4], a process which is thought to involve membrane fusion events [5,6]. The capacity of such membranes to fuse in the presence of GTP may therefore be an essential feature within all eukaryotic cells. In this paper we examine the tissue and species specificity of GTP-stimulated membrane fusion. It is shown that GTP can promote fusion between homologous nuclear membranes derived from various sources of tissues and animals as well as between heterologous nuclear membranes following mixing of different types of nuclei.

Materials and Methods

Materials. 150–200 g Sprague-Dawley rats were obtained from the Charles River Canada Inc. Laboratories (St. Constant, Quebec), quails (*Coturnix coturnix japonica*) were from La Ferme du Gourmet (Beloeil, Quebec), and frogs (*Rana catesbeiana*) were from La Firme Cimon (Loretteville, Quebec). Glutaraldehyde was from Polysciences, Inc. (Warrington, PA, U.S.A.) and osmium tetroxide from Meca Laboratories (Pointe-aux-Trembles, Quebec). Adenosine triphosphate (ATP), guanosine triphosphate (GTP), phospho*enol*-

pyruvate, and pyruvate kinase were all from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were from Canadian Laboratory Supplies (Canlab, Montreal, Quebec) and were of the highest purity available.

Preparation and treatment of nuclear fractions. Nuclei were prepared using the method of Blobel and Potter [7] from rat liver homogenates (1 : 3 w/v in buffer I, e.g., 0.25 M sucrose/50 mM Tris-HCl (pH 7.5)/25 mM KCl/5 mM $MgCl_2$). Nuclear fractions were obtained from rat brain and quail liver homogenates using the same procedure. Nuclei from frog liver were obtained by differential centrifugation [8] using buffer I as homogenizing medium.

Incubation of nuclei. Unless otherwise stated, 0.2 ml of washed nuclei ($(2–9)\cdot 10^7$ nuclei in buffer I, employed immediately after resuspension) were incubated at 37°C (15–120 min) with 0.3 ml of a buffer containing 66.66 mM Tris-HCl (pH 7.4), 50 mM KCl, 12.5 mM $MgCl_2$, 4.17 mM $MnCl_2$, 1.67 mM ATP, 0.84 mM GTP, 16.67 mM phospho*enol*pyruvate and 25 μg pyruvate kinase. This mixture comprised the standard medium. Control incubation mixtures were the same except GTP was omitted.

Electron microscopy

After incubation, nuclei were fixed for thin-section electron microscopy with 2.5 ml of ice-cold glutaraldehyde (1.5% in 0.05 M phosphate buffer (pH 7.4)). After fixing overnight, the nuclei were recovered by filtration onto Millipore membranes (Millipore Corp., Bedford, MA) using the random filtration method of Baudhuin [9] as previously described [1]. Nuclei were then bloc-stained, dehydrated and processed for electron microscopy [1].

Assay for fusion. Fusion between outer nuclear membranes was monitored by a morphological assay previously described [1]. This assay permits determination of the formation of fusion products. When isolated nuclei are incubated in vitro in the presence of GTP the nuclei aggregate. The outer membranes which make contact fuse along the periphery of adjoining nuclei. The fusion proceeds along the surface of the nuclei and between the regions of the nuclear pores in such a way that the resulting fusion products (contact zones between fused nuclear pairs) can be easily identified in the electron microscope. The contact zones are delimited by two continuous outer membranes, contain confluent perinuclear spaces and have small vesicles with ribosomes in their lumen which often interconnect the fused nuclei at the level of nuclear pores (Fig. 1). Because the incidence of sectioning through a contact zone between a fused nuclear pair can vary considerably, occasionally, one of the components of such a zone (e.g., continuous outer membrane, confluent perinuclear space or inverted vesicles with ribosomes in their lumen) is not well represented in the photomicrography.

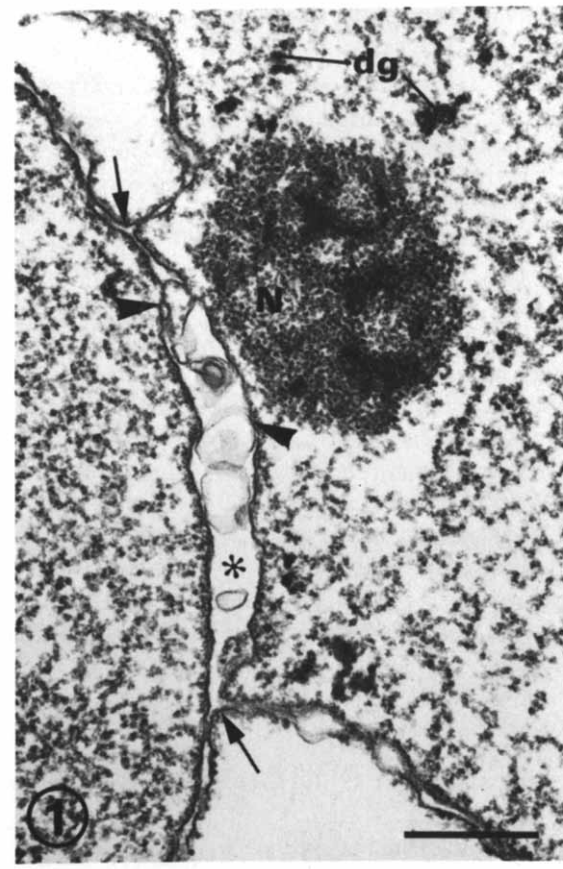


Fig. 1. Electron micrograph of rat liver nuclei after incubation in standard medium plus GTP for 120 min at 37°C. The contact zone between nuclei showing typical 'fusion' characteristics is shown. Such a contact zone is comprised of outer membranes continuous over adjacent nuclei (arrows), confluent perinuclear spaces (asterisk) and vesicles joining adjacent nuclei at the level of their respective inner membranes (arrowheads). The dense granules (dg) are similar to those described previously within incubated nuclei [10]. N, nucleolus. Bar, 0.5 μm. Magnification, ×35000.

However, the presence of the other two components is still evident and allow positive identification of the fused nuclear pairs.

The formation of fused nuclear pairs can be quantitated [1]. This is done by counting closely aggregated nuclear pairs and determining the percent number of pairs which have the morphological properties described above for nuclei whose outer membranes have become continuous as a result of membrane fusion.

Results

Effect of GTP on the fusion between homologous nuclear membranes

Using the quantitative fusion assay described in the Materials and Methods the time and temperature dependence of the fusion of the outer membranes of liver nuclei was determined (Fig. 2, Table I). At 37°C, this phenomenon was already detectable after 15 min, increased steadily till 60 min and started to plateau at around 120 min (Fig. 2). Incubation of rat liver nuclei in the presence of GTP at various temperatures revealed that outer nuclear membrane fusion was highly temperature dependent (Table I). No paired nuclei (or very few) were observed with continuous outer membranes at temperatures below 20°C indicating that very little fusion occurred at these temperatures. At temperatures higher than 20°C the amount of fusion observed was considerable, and remarkably similar as indicated by the number of nuclear pairs identified with continuous outer membranes (Table I).

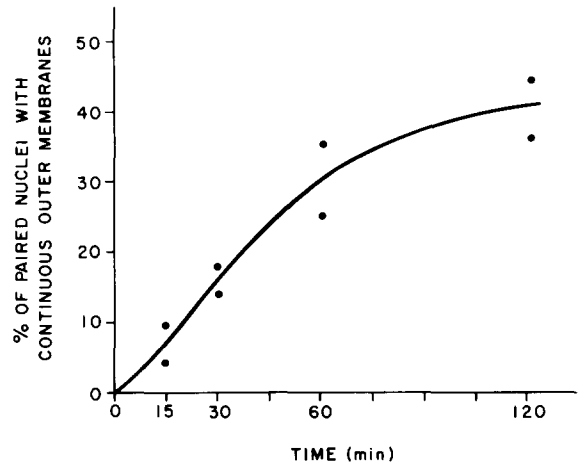


Fig. 2. Time-course of GTP-stimulated formation of paired nuclei with continuous outer membranes. Rat liver nuclei were incubated in the standard medium containing 0.5 mM GTP at 37°C for various periods of time. Quantitation was carried out as previously described [1]. The values represent data obtained from two separate experiments. For each time point greater than 100 pairs of nuclei were counted and the number of pairs with continuous outer membranes was expressed as a percentage of the total.

Evidence of nuclear membrane fusion was observed when various types of nuclei were incubated in the presence of GTP, these include nuclei from rat brain (Fig. 3, Table II) quail liver (Fig. 4, Table II) and frog liver (Fig. 5). When such nuclei were incubated in the absence of GTP, they aggregated but did not become connected by fused outer membranes (not shown).

The rat brain nuclear fraction was composed of

TABLE I

EFFECT OF TEMPERATURE ON GTP-STIMULATED FORMATION OF PAIRED NUCLEI WITH CONTINUOUS OUTER MEMBRANES

Rat liver nuclei were incubated at various temperatures for 120 min in the presence of standard medium plus 0.5 mM GTP. Quantitation was done as in Fig. 2. Values in parentheses represent results of a separate experiment.

Incubation temperature (°C)	No. of paired nuclei	No. of paired nuclei with continuous outer membranes	% of paired nuclei with continuous outer membranes
4	111 (101)	0 (0)	0
10	99 (102)	2 (6)	4
20	68 (97)	29 (39)	41.2
30	103 (103)	43 (51)	45.6
37	101 (103)	41 (48)	43.6

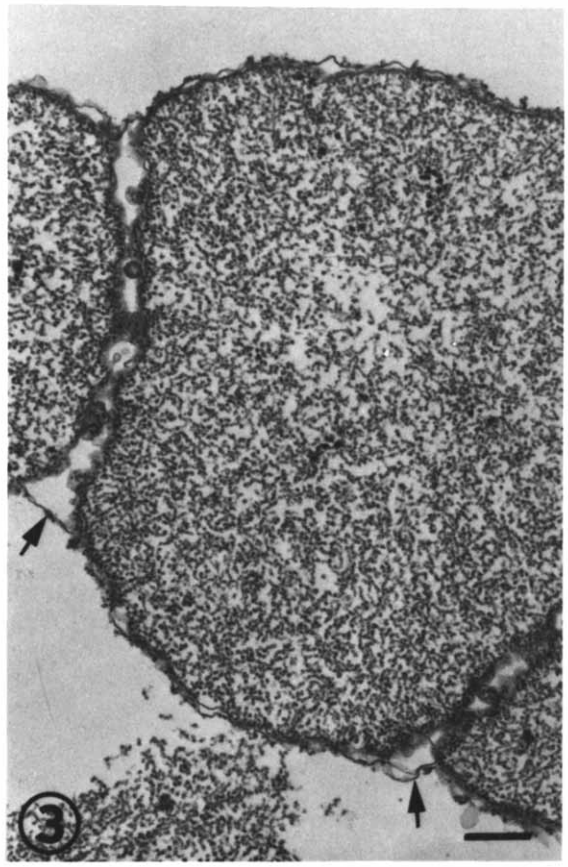


Fig. 3. Brain nuclei incubated 120 min at 37°C in standard medium plus GTP. Zones of close nuclear apposition are shown between three nuclei which contain chromatin of identical morphological characterisitics. The fused outer membranes are shown (arrows) between nuclei. Bar, 0.5 μm. Magnification, ×20000.

a heterogeneous population of nuclei. The majority of nuclei showed a diffuse chromatin pattern had small nucleoli and were often observed fused together after incubation in the presence of GTP (Fig. 3). A small proportion of nuclei revealed a condensed chromatin pattern, had larger more prominent nucleoli and were occasionally observed interconnected by fused outer membranes after incubation in the presence of GTP (not shown). Although heterologous nuclei were occasionally observed in close contact (not shown) none were observed interconnected by a fused outer membrane. This prompted questions about the cell specificity of the fusion phenomenon being

TABLE II

EFFECT OF INCUBATING HETEROLOGOUS NUCLEI IN THE PRESENCE OF GTP ON THE FORMATION OF PAIRED NUCLEI WITH CONTINUOUS OUTER MEMBRANES

Different combinations of nuclei were incubated in the presence of 0.5 mM GTP at 37°C under slightly varying incubation conditions (see text for details). Quantitation was done as in Fig. 2. Greater than 100 nuclear pairs with continuous outer membranes were counted for each combination.

Nuclear combination	% of total nuclear pairs with continuous outer membranes	
	Homologous	Heterologous
Rat liver	48.8	
+		20.9
Rat brain	30.2	
Rat liver	31.8	
+		30.3
Quail liver	37.8	
Quail liver	36.5	
+		38.1
Rat brain	25.4	

examined. Therefore nuclei were isolated from different tissues and different species of animals and were incubated as mixtures in the presence of GTP to see if heterologous nuclear membranes could indeed fuse together to form heterologous membrane hybrids.

Effect of GTP on the fusion between heterologous nuclear membranes

The effect of various incubation parameters on nuclear structure has previously been described [10]. Under normal conditions homologous nuclei were incubated 120 min at 37°C in standard medium. However, for heterologous nuclei incubation conditions were varied slightly in order to maximize morphological differences amongst the nuclei and to ensure proper identity of nuclei in mixed populations. Preliminary studies (not shown) indicated that for certain heterologous nuclear mixtures long incubation periods (e.g. greater than 60 min) led to morphological transformations which made it difficult to identify heterologous nuclear pairs therefore in some instances incubations were carried out for shorter periods of time. In other cases incubation of heterologous mixtures

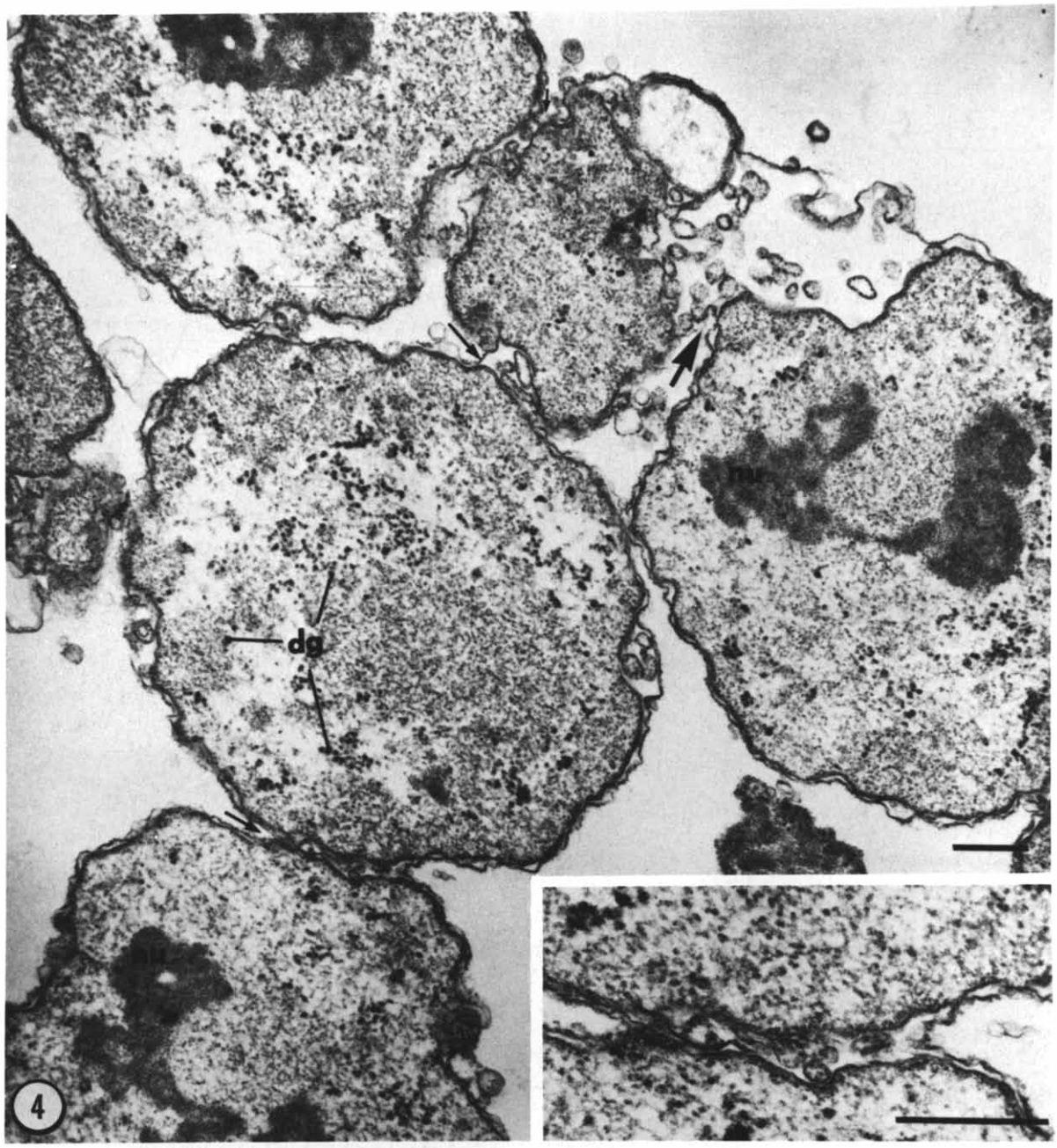
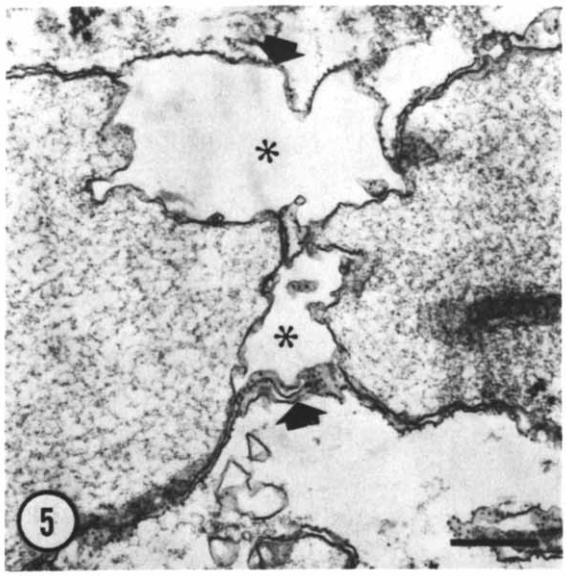


Fig. 4. Quail liver nuclei after incubation in the presence of GTP in standard medium at 37°C for 120 min. The typical polymorphic structure of the quail liver nucleoli (nu) is evident in two of the nuclei, all nuclei have diffuse chromatin and aggregated dense granules (dg). Contact zones with fused outer nuclear membranes are indicated (arrows). Bar, 0.5 μm. Magnification, ×22500. The inset reveals more detail of one of these contact zones. A continuous outer membrane can be distinguished as well as confluent perinuclear spaces. Small vesicles between apposed inner nuclear membranes can also be seen. Bar, 0.5 μm. Magnification, ×45000.

were done in the absence of a nucleotide regenerating system. It was previously shown [10] that such incubations led to marked changes in chromatin structure in incubated nuclei. Preliminary work (not shown) indicated that omission of the nucleotide regenerating system in the incubation



medium of heterologous mixtures accentuated certain morphological differences between nuclei of different origins.

Mixtures of nuclei from different tissues of the same species. When rat brain nuclei were incubated in the presence of liver nuclei and GTP, aggregates of nuclei were formed containing homologous as well as heterologous nuclear pairs. The two types of nuclei were recognized based on their different morphologies after incubation in standard medium minus pyruvate kinase and phospho*enol*pyruvate for 30 min at 37°C (Fig. 6). Using these particular conditions of incubation rat

Fig. 5. Frog liver nuclei after incubation in standard medium for 120 min at 25°C. The pair of nuclei are joined by continuous outer membranes (arrows) and confluent perinuclear spaces (*). Bar, 0.5 μm. Magnification, ×22500.

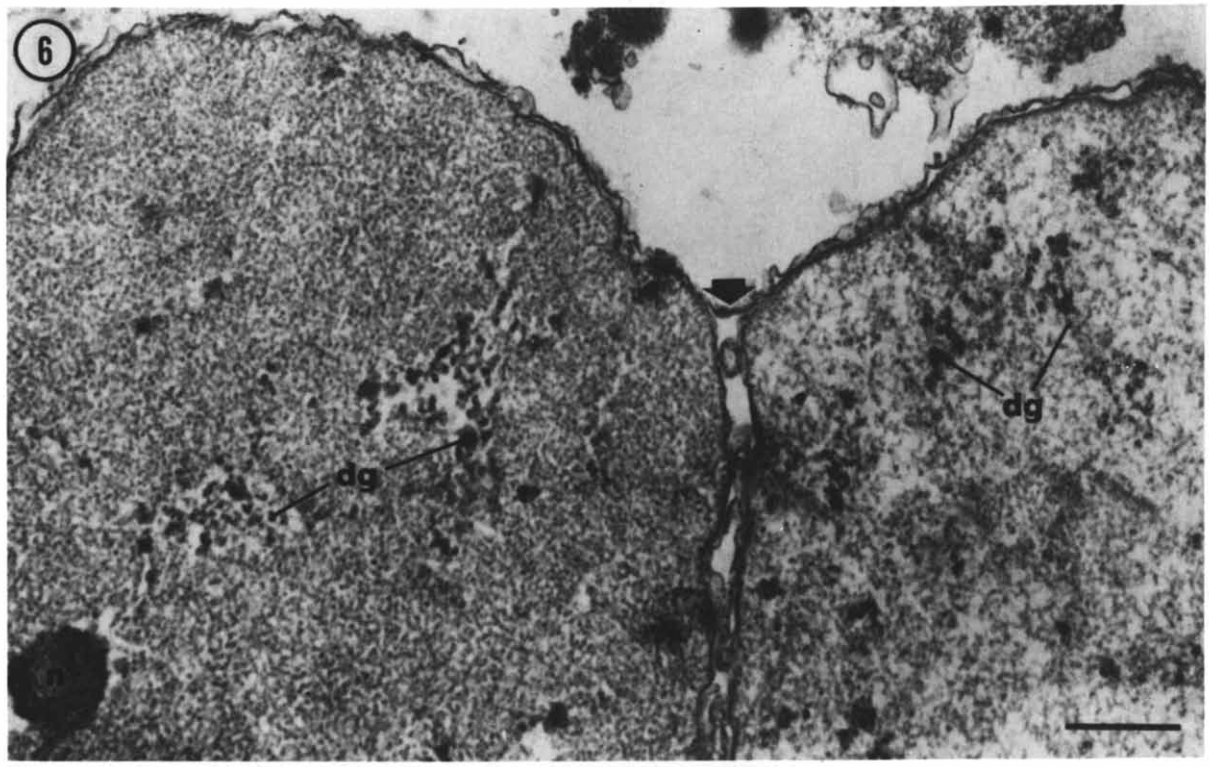


Fig. 6. Contact zone between a rat brain nucleus (left) and a rat liver nucleus (right) after incubation for 30 min at 37°C in the presence of GTP but absence of phospho*enol*pyruvate and pyruvate kinase. The outer membranes of both nuclei are continuous (arrow). The perinuclear spaces of both nuclei are confluent in the zone of contact and small vesicles interconnect the two nuclei at the level of the respective inner membranes. Note the difference between the granular aspect of the rat brain nuclear chromatin (left) and the diffuse filamentous nature of the rat liver nuclear chromatin (right). The dense granules (dg) of the rat brain nucleus are highly clumped and surrounded by electron luscent areas. The dense granules of the rat liver nucleus are less aggregated and contrast less with their surrounding luscent areas. n, nucleolus. Bar, 0.5 μm. Magnification, ×30000.

liver nuclei could be distinguished by the fact that they contained diffuse filamentous chromatin, a homogeneous distribution of electron dense granules (ribonucleoprotein particles [10]) and large prominent nucleoli. The rat brain nuclei were different they were smaller, contained a more granular chromatin, a heterogeneous distribution of electron dense granules and small nucleoli. Both homologous (Table II) and heterologous pairs (Fig. 6, Table II) were observed joined by continuous outer membranes as a result of fusion in the presence of GTP. When GTP was omitted from the incubation medium aggregates of nuclei were still formed but neither homologous nor heterologous nuclear pairs were joined by fused outer membranes (not shown).

Mixtures of nuclei from same tissues of different species. Mixtures of nuclei obtained from rat liver and quail liver were incubated in standard medium minus pyruvate kinase and phospho*enol*pyruvate in the presence or absence of GTP for 60 min. In the absence of GTP nuclear aggregates were formed but no pairs of nuclei were observed joined by fused outer membranes (not shown). The two types of nuclei were recognized after incubation based on their respective morphologies (Fig. 7). The rat liver nuclei contained condensed perinuclear and condensed perinucleolar chromatin, dense granules associated with the condensed chromatin and prominent round or oval nucleoli. The quail liver nuclei contained diffuse chromatin, dense granules dispersed throughout the nucleoplasm and large polymorphic nucleoli (the latter characteristic is typical of quail nucleoi [11]). When mixtures of nuclei were incubated as above and in the presence of GTP aggregates were formed and

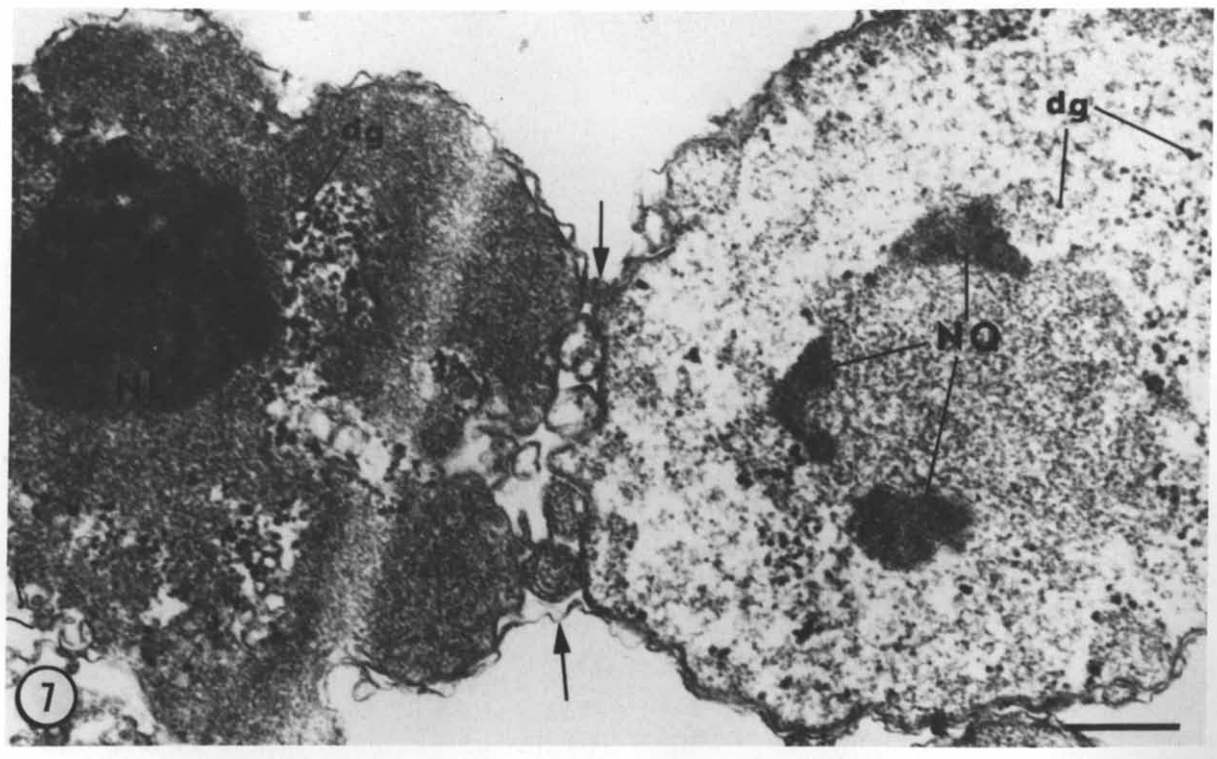


Fig. 7. Mixture of rat liver and quail liver nuclei after incubation for 60 min at 37°C in standard medium containing GTP but lacking pyruvate kinase and phospho*enol*pyruvate. A rat liver nucleus is evident on the left with condensed perinuclear and perinucleolar chromatin, dense granules (dg) closely apposed to the condensed chromatin, and an oval nucleolus (NL). A quail liver nucleus, on the right is different having a diffuse chromatin pattern, randomly distributed dense granules and a typical polymorphic nucleolus (NQ). Note that the contact zones between the heterologous pair is typical for fused nuclei. Continuous outer membranes can be seen (arrows) as well as confluent perinuclear spaces. Vesicles are interspersed within the contact zone between the nuclei. Bar, 0.5 μm. Magnification, $\times$30000.

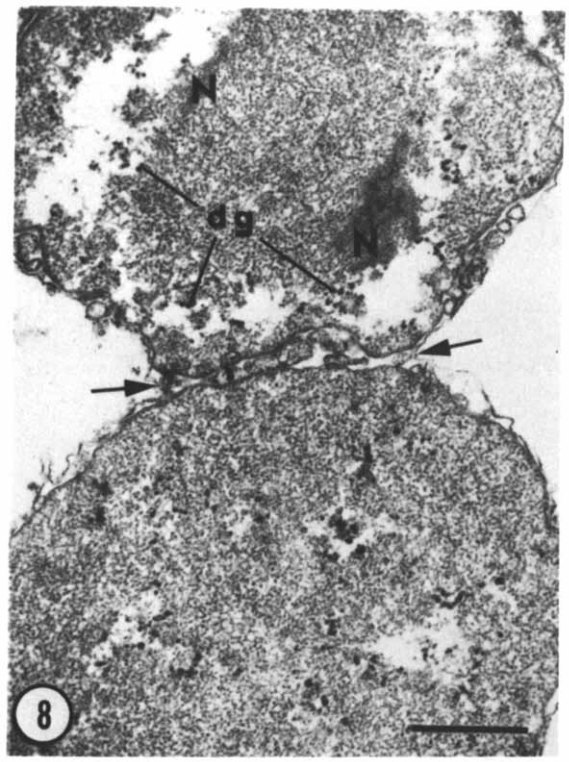


Fig. 8. A heterologous nuclear pair after incubation for 60 min at 37°C in standard medium containing GTP but lacking pyruvate kinase and phospho*enol*pyruvate. A quail liver nucleus is observed above with a central core of chromatin delimited by numerous small dense granules (dg) and nucleolar fragments (N). The nucleus below represents a rat brain nucleus showing granular chromatin and dispersed dense granules. Continuous outer membranes (arrows) join the pair of nuclei along the periphery of their contact zone. Bar, 0.5 μm. Magnification, ×30000.

both homologous (Table II) as well as heterologous pairs (Fig. 7, Table II) became interconnected by a fused outer membrane.

Mixtures of nuclei from differeent tissues of different species. Nuclei obtained from rat brain were incubated in the presence of nuclei derived from quail liver in standard medium lacking pyruvate kinase and phospho*enol*pyruvate plus or minus GTP. Incubation led to the formation of nuclear aggregates amongst which could be recognized nuclear pairs with either similar or dissimilar morphologic properties. Such nuclear pairs were never observed with continous outer membranes when incubation was carried out in the absence of GTP. However when mixtures of nuclei were incubated in the presence of GTP both homologous (Table II) and heterologous nuclear pairs (Fig. 8, Table II) were observed joined by fused outer membranes.

Discussion

The results show that homologous nuclear membranes from different tissues and species of animals can fuse when incubated in vitro in the presence of GTP. This supports the previous suggestion that GTP-dependent fusion may be a property common to most if not all eukaryotic nuclear membranes [1]. The capacity for nuclear membranes to fuse in the presence of GTP is therefore considered a property which has been highly conserved during the evolution of the cell. This fact as well as the fact that nuclear membrane fusion occurs at physiological concentrations of GTP (Refs. 1, 4 and this paper) gives further support to the possibility that this phenomenon could have important biological significance.

Membrane fusion has been implicated in nuclear envelope assembly in post-mitotic cells [5,6]. Our present working hypothesis is that GTP serves as an important regulator of the fusion events involved in nuclear envelope biogenesis [4]. The high nucleotide [1] and membrane [4] specificities of this phenomenon as well as the low species specificity (this paper) are consistent with the above proposal. High nucleotide specificity would favor nuclear membrane interaction in post-mitotic cells at a time when there is high mobilization of free nucleotide pools [12]. High membrane specificity would ensure interactions only between nuclear envelope and/or endoplasmic reticulum derivatives in a cell which contains a mixture of different membrane types, all having equal opportunity of making contact and interacting. Finally low tissue and species specificity would allow interaction between nuclear membranes of such diverse cell types as the male and female gametes following gamete fusion [13,14,15]. Of direct relevance here is the previous demonstration that fusion may occur between the pronuclei of the gametes of two species as varied as the sea urchin (*Arbacia*) and the common mussel (*Mytilus*) [16].

GTP-dependent fusion occurs between heterol-

ogous nuclear membranes. This constitutes the first in vitro demonstration of physiologically stimulated fusion between heterologous membranes at the intracellular level. The data suggests that cell specific membrane recognition factors probably do not play a role in controlling interactions between membranes of this particular type. However, other factors (genetically determined) such as the ribosome density or density of recognition sites along the outer membranes, might influence such interactions. For example the high density of ribosomes along the outer membranes of certain nuclei would be expected to be a hindrance to membrane contact and consequently prevent fusion. Low densities of recognition sites along the outer membranes of certain nuclear preparations might affect their chances of establishing firm and stable contacts with other nuclear species and thus decrease the probabilities of forming fused heterologous pairs. Such factors may have been responsible for the lack of fusion between heterologous nuclei occasionally observed in rat brain nuclear preparations and alluded to in the results. However, without a systematic analysis of the effects of such factors on formation of fused heterologous nuclear pairs these suggestions remain essentially speculative ones.

The mechanism by which GTP can stimulate membrane fusion is unknown. moreover, it has not been demonstrated whether GTP can stimulate membrane fusion in vivo. Experiments are presently underway to examine such questions.

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