Amplification of a Specific Set of Intracisternal A-Particle Genes in a Mouse Plasmacytoma

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Intracisternal A-particles (IAPs) derive from endogenous retrovirus-like genes that are present in ca. 1,000 copies per haploid mouse genome. A particular IAP sequence variant that represents only 2% of the endogenous genes was found to be the predominant transcript in five out of seven plasmacytoma lines examined, and these genes have been amplified two- to fourfold in MOPC 315. Restriction enzyme digests of normal and tumor DNAs revealed a set of conserved sites within the population of amplified genes which could be used to generate a consensus restriction enzyme map of the amplified IAP genome. Molecular clones of IAP genes related to those amplified in MOPC 315 were isolated from gene libraries of MOPC 315 and MOPC 104E DNAs. Each clone was characterized for the presence of the restriction enzyme sites conserved in the amplified genes. Of 26 clones isolated from MOPC 315 DNA, 14 contained all of the conserved sites, whereas none of the clones isolated from MOPC 104E DNA contained all of the sites. These results suggested that approximately one-half of the genes isolated from MOPC 315 DNA were members of the amplified IAP gene population, representing newly acquired proviruses in the tumor DNA. Unique cellular flanking sequences were isolated from four of the MOPC 315 clones and four of the MOPC 104E clones. Probes from three of the MOPC 315 clones detected restriction fragments that were unique to MOPC 315 DNA and equivalent in size to the cloned gene, demonstrating that these three IAP genes had integrated into new sites in the plasmacytoma DNA. The other five clones were found to be endogenous to the BALB/c genome. Thus, IAPs represent a significant source of insertional mutations for any cell expressing the retrovirus-like particles. Furthermore, these results suggest that only one or a small number of endogenous IAP sequences are activated to give the homogeneous population of amplified genes.

Intracisternal A-particles (IAPs) are endogenous retrovirus-like structures found abundantly in many mouse tumor cells (7, 9, 11) but only rarely in normal cells (23). IAPs derive from a set of nearly 1,000 endogenous genes which are interspersed throughout the mouse genome (15, 17). We have been interested in the relationship between IAP activation and tumor formation and, in particular, whether or not specific IAP genes are activated in tumor cells and whether the cytoplasmic particles can reintegrate into new sites in the mouse genome.

Previous studies have shown that IAP RNAs differ among tumor types. A 7-kilobase (kb) IAP gene transcript was found in a neuroblastoma line (18), whereas the predominant RNA in two plasmacytoma lines is a 3.8-kb transcript from a somewhat different IAP gene (17). This suggests that different populations of IAP genes are active in the two tumor types. Furthermore, the active genes in the plasmacytomas represent only a small fraction (20 genes, or 2%) of the endogenous IAP sequences (20), raising the possibility that only one or very few genes are transcribed in any given tumor. An investigation into the chromosomal domains at which these activated genes reside could provide important information about gene expression in transformed cells.

Another consequence of IAP gene activation is the potential for reintegration of proviral sequences into the mouse genome. It has been demonstrated for exogenous viruses that proviral integration can activate cellular oncogenes by promoter insertion (12). Recent studies suggest that IAP proviruses can also alter the expression of cellular genes. For example, immunoglobulin C_κ genes were found to be inactivated by IAP integration in a hybridoma line (14), and the *c-mos* oncogene was found to be activated by IAP

insertion in a plasmacytoma line (13). Finally, a previous study from this laboratory described a two- to fourfold amplification of an IAP gene family in two plasmacytoma lines (20). This gene amplification was presumably due to integration of intracellular viral genes into new sites in the tumor DNA. Thus, IAP genes can activate, inactivate, or potentially alter cellular gene expression at many sites in tumor cells.

In this study, the amplified IAP genes in MOPC 315 have been characterized for the presence of conserved restriction enzyme sites and the locations of the proviruses in the tumor genome. The results suggest that the amplified genes may derive from only a single endogenous IAP sequence. Three proviruses were identified which have integrated at new sites in the tumor DNA.

MATERIALS AND METHODS

Isolation of liver and plasmacytoma DNA and RNA was as previously described (17, 20). DNA fragments for nick translation were purified from low-melting-point agarose by a method provided by Bethesda Research Laboratories. Electrophoresis of restriction enzyme-digested DNA and transfer to nitrocellulose were carried out as described (17). Filter hybridization was performed in a buffer described previously (20), which was supplemented with 10% (wt/vol) dextran sulfate (22) for experiments involving total mouse DNA digests. Polyadenylic acid [poly(A)]-containing RNA was electrophoresed on formaldehyde-agarose gels (19) with a sodium borate buffer (10) and transferred to nitrocellulose as described previously (21). Hybridization was carried out as for the DNA blots above.

IAP clones were isolated from gene libraries of partial *EcoRI*-digested tumor DNAs with the Charon 4A vector system (4). Clones were selected by the plaque lift technique

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FIG. 1. Diagram of the two types of IAP genes. The sequence variants of type II genes are shown, as well as the segment subcloned into pBR322 for use as a type II-specific probe. The crosshatched area between the long terminal repeat (LTR) and the type II-specific sequence is common to both type I and type II genes as indicated (17). The restriction enzyme sites indicated are: E, *EcoRI*; H, *HindIII*; and S, *SstI*.

(2) after hybridization with the type II IAP probe described below. DNA from individual positive plaques was prepared by a rapid plate lysate protocol (8), digested with the appropriate restriction enzymes, and analyzed by Southern blotting with the type II probe. Clones containing a 2.8-kb *EcoRI-HindIII* fragment were further plaque purified and grown in large-scale preparations. Isolated restriction fragments were subcloned into pBR322 as indicated.

The type II-specific sequence was subcloned in pBR322 from two previously described IAP genes (17). Clone 19 is a type IIA gene with a 3.5-kb *EcoRI-HindIII* fragment, whereas clone 62 has a 1.6-kb deletion that begins at the 3' boundary of the type II-specific sequence. The two IAP genes (as *EcoRI* fragments cloned into pBR322) were cleaved with *PstI*, denatured, reannealed, and digested with *S1* nuclease, which cleaves at the deletion loop in the heteroduplex. A 4.5-kb DNA fragment that contained pBR322 linked to the leftmost 0.9-kb portion of the IAP gene was isolated, ligated to *Bam*HI linkers, partially digested with *Bam*HI, self-ligated, and transfected into HB101. The leftmost 0.5-kb portion of the IAP genome was excised from the preceding clone by *EcoRI* and *SstI* cleavage, digested with *S1* nuclease, self-annealed, and transfected into HB101. The resultant plasmid contains the 0.5-kb segment of the IAP gene that is specific for type II genes (Fig. 1).

RESULTS

Previous studies have shown that the predominant IAP RNAs in two different plasmacytoma lines are colinear with a minor population of IAP genes (20). We have designated these genes type IIB, and they can be distinguished by a 0.7-kb deletion when compared with the larger type IIA genes (Fig. 1). Ten percent of the endogenous IAP sequences in the mouse genome are type II genes, and type IIB genes are only present in ca. 20 copies, or 2%. To study these actively expressed genes in more detail, a subclone was constructed that contained only the type II-specific 0.5-kb sequence identified previously (Fig. 1). The method by which this sequence was cloned is outlined above. This type II-specific DNA segment was used as a probe for all of the experiments described below.

Type IIB transcripts in four plasmacytoma lines. It was initially important to determine whether type IIB genes were transcribed in only some or all plasmacytomas. Poly(A)-containing RNAs were isolated from seven different tumor

lines, electrophoresed on formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized to the type II probe (Fig. 2). In four of the seven lines, an abundant 3.8-kb RNA species was found which was equivalent in size to the type IIB transcript previously described (17). Fainter bands at 4.2 kb were evident in MOPC 46B and MOPC 104E. Hybridization with other IAP probes which detect type I IAP genes showed a much lower level of 7-kb type I transcripts in all tumors as described previously (17). W3469 had only very low levels of IAP RNA, which were visible with longer exposure. This is consistent with the previous observation that this tumor line has few IAPs (1). However, the most abundant transcript was the same 3.8-kb type IIB RNA (data not shown). There were no type II transcripts in MOPC 460 and RPC20. Thus, in five out of seven plasmacytoma lines, type II IAP RNAs of the same size as those previously characterized were abundantly transcribed.

Characterization of amplified type IIB genes in MOPC 315. MOPC 315 and MOPC 104E DNAs have been shown previously to have an enhanced 2.8-kb *EcoRI-HindIII* IAP band that corresponds to the type IIB genes that are actively transcribed in those tumors (20). This presumably represents an amplification of the type IIB genes in the tumor DNA by reintegration of the cytoplasmic IAP genomes. To characterize these amplified IAP genes further, MOPC 315, MOPC 104E, and BALB/c DNAs were digested with different restriction enzymes and hybridized to the type II probe (Fig. 3). With *EcoRI* and *HindIII*, there were three bands of hybridization, two of which correspond to the previously

FIG. 2. Type II IAP RNAs in mouse plasmacytomas. Poly(A)-containing RNAs (5 μ g) from seven plasmacytoma lines were electrophoresed on formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized to the type II-specific probe. The size of the prominent 3.8-kb RNA was measured from denatured *HindIII*-digested DNA run in parallel.

FIG. 3. Southern blot analysis of the amplified, type IIB IAP genes in MOPC 315. (A) Different restriction enzyme digests of BALB/c (1), MOPC 104E (2), and MOPC 315 (3) DNAs were electrophoresed on a 0.6% agarose gel, transferred to nitrocellulose, and hybridized with the type II-specific probe. The size of the amplified band in MOPC 315 is indicated for each digest. The restriction enzymes used were: E, *EcoRI*; H, *HindIII*; B, *BamHI*; S, *SstI*; X, *XbaI*; M, *MspI*; and P, *PstI*. (B) Consensus restriction enzyme map of the amplified IAP gene in MOPC 315 based on the data in (A) and additional mapping experiments with cloned genes (not shown). LTR, Long terminal repeat.

described type IIA and IIB genes. An additional band of hybridization at 2.3 kb represents a third sequence variant of the type II genes (IIC) with a more extensive deletion analogous to the type IIB variant (data not shown). The 3.5-kb and 2.3-kb *EcoRI-HindIII* bands were reproducibly broader than the band at 2.8 kb. This arises from restriction fragment length heterogeneity among individual genes of the type IIA and IIC groups, which is not found in the type IIB genes (data not shown).

As described previously, the 2.8-kb *EcoRI-HindIII* band (type IIB) was significantly enhanced in MOPC 315 and somewhat enhanced in MOPC 104E. Enhanced hybridization was also found at 2.0 kb with *EcoRI* and *BamHI*, 2.4 kb with *SstI* and *HindIII*, 1.0 kb with *XbaI*, 1.6 kb with *MspI*, and 3.5 kb with *PstI*. Each of these enzymes apparently recognizes a conserved site(s) within the amplified type IIB genes which gives rise to the enhanced hybridization. From these data, a consensus restriction enzyme map of the amplified type IIB genes could be deduced (Fig. 3B). These results suggested that the transcribed gene(s) in MOPC 315 should have all of the conserved restriction enzyme sites in this consensus map. In many of the digests, MOPC 104E DNA did not have noticeably more hybridization than did BALB/c DNA for the bands that were clearly amplified in MOPC 315. It is not known if this is due to a different pattern of conserved sites in amplified MOPC 104E genes or if the previous estimate of the extent of amplification in MOPC 104E was inaccurate. One observation that would support the former suggestion is that there is a reproducible difference in the mobility of the type IIB RNA between MOPC

315 and MOPC 104E (Fig. 2). This may reflect a different type IIB IAP gene(s) transcribed in the two tumors.

Molecular cloning of type IIB genes. The results presented above were most consistent with type IIB IAP genes integrated at many new sites in MOPC 315 tumor DNA. To demonstrate this directly, many type IIB genes were cloned from phage libraries of MOPC 104E and MOPC 315 DNAs. Primary positive plaques were screened for the presence of a 2.8-kb *EcoRI-HindIII* fragment by a rapid plate lysate protocol, followed by restriction and hybridization to the type II probe. Type IIB clones were plaque purified and characterized for the presence of the conserved restriction enzyme sites from the amplified genes in MOPC 315 (Fig. 3B). Tables 1 and 2 show the results of the analysis of 25 clones from a MOPC 104E library and 26 clones from a MOPC 315 library. All clones were also characterized for the size of the *EcoRI* fragment that contained the IAP gene and adjacent cellular flanking sequences so that the individual isolates could be compared for possible duplication.

From the tables, it is clear that there are significant differences between the two populations of genes. In MOPC 104E, all of the clones lack one or more of the consensus restriction enzyme sites from the amplified genes in MOPC 315. However, in the MOPC 315 clones, 14 out of 26 clones have all the consensus sites tested. This is most striking for the *MspI* band of 1.60 kb in the MOPC 315 genes. This band falls slightly below a broad band of hybridization in BALB/c DNA, indicating that there are very few type II genes with a 1.60-kb *MspI* fragment in the BALB/c genome (Fig. 3). Yet, the 1.6-kb *MspI* fragment is present in 16 out of the 26

TABLE 1. Summary of restriction enzyme mapping of MOPC 104E type IIB IAP clones

λ -Clone	Detection of conserved fragments with type II sequence ^a						Size of M-M fragment (kb)	No. of conserved sites/no. of enzymes tested
	E-H (2.8)	E-B (2.0)	S-H (2.4)	X-X (1.0)	M-M (1.6)	E-P (3.1)		
15-104E	+	+	+	+	—	—		5/7
45-104E	+	+	—	—	—	—		3/7
52-104E	+	+	+	—	—	—		4/7
55-104E	+	+	—	—	—	+	1.35	4/7
57-104E	+	+	+	+	—	+	1.35	6/7
65-104E	+	—	—	—	—	—		2/7
76-104E	+	—	—	—	—	—		2/7
98-104E	+	+	—	—	—	+	1.35	4/7
120-104E	+	—	+	+	—	—		4/7
122-104E	+	—	+	ND	—	—		3/6
L72-104E	+	+	+	+	—	+	1.35	6/7
L74-104E	+	+	—	+	—	+	1.50	5/7
L75-104E	+	+	+	+	—	—	1.35	5/7
L76-104E	+	+	+	+	—	—	1.35	5/7
202-104E	+	+	—	—	—	—		3/7
244-104E	+	+	+	+	—	+	1.35	6/7
258-104E	+	+	+	+	—	+		6/7
266-104E	+	+	—	ND	ND	+		4/6
269-104E	+	+	—	—	ND	+		4/6
280-104E	+	+	—	—	—	+	1.50	4/7
281-104E	+	+	+	—	—	+	1.55	5/7
294-104E	+	+	+	+	—	+		6/7
344-104E	+	+	—	—	—	—		3/7
350-104E	+	+	+	+	—	+	1.35	6/7
356-104E	+	+	+	+	—	—	1.35	5/7

^a Symbols: E, *EcoRI*; H, *HindIII*; B, *BamHI*; S, *SstI*; X, *XbaI*; M, *MspI*; P, *PstI*. Numbers in parentheses represent kb. ND, not determined.

TABLE 2. Summary of restriction enzyme mapping of MOPC 315 type IIB IAP clones

λ -Clone	Detection of conserved fragments with type II sequence ^a						Size of M-M fragment (kb)	No. of conserved sites/no. of enzymes tested
	E-H (2.8)	E-B (2.0)	S-H (2.4)	X-X (1.0)	M-M (1.6)	E-P (3.1)		
14-315	+	+	+	+	+	ND	1.60	6/6
15-315	+	+	+	+	+	ND	1.60	6/6
16-315	+	+	+	+	+	ND	1.60	6/6
38-315	+	+	+	+	+	+	1.60	7/7
39-315	+	+	—	ND	—	ND	1.35	3/5
41-315	+	+	+	ND	—	ND	1.50	4/5
53-315	+	+	+	ND	—	ND	1.40	4/5
62-315	+	+	+	+	—	ND	1.50	5/6
68-315	+	+	+	+	—	ND	1.40	5/6
75-315	+	+	+	ND	+	ND	1.60	5/5
76-315	+	+	+	+	+	+	1.60	7/7
86-315	+	+	+	ND	+	ND	1.60	5/5
88-315	+	+	—	ND	—	ND	1.50	3/5
98-315	+	+	+	+	+	+	1.60	7/7
99-315	+	+	+	—	+	ND	1.60	5/6
103-315	+	+	+	+	+	ND	1.60	6/6
107-315	+	+	+	+	—	ND	1.35	5/6
123-315	+	+	+	+	+	ND	1.60	6/6
131-315	+	+	—	—	—	ND	1.50	3/6
149-315	+	+	+	ND	+	ND	1.60	5/5
150-315	+	+	+	ND	+	ND	1.60	5/5
154-315	+	+	+	+	+	ND	1.60	6/6
162-315	+	+	+	+	—	ND	1.50	5/6
168-315	+	+	—	—	+	ND	1.60	4/6
172-315	+	+	—	ND	—	ND	1.35	3/5
179-315	+	+	+	+	+	+	1.60	7/7

^a Symbols: E, *EcoRI*; H, *HindIII*; B, *BamHI*; S, *SstI*; X, *XbaI*; M, *MspI*; P, *PstI*. Numbers in parentheses represent kb. ND, not determined.

MOPC 315 clones, whereas it was not found in any of the MOPC 104E genes.

Newly acquired IAP proviruses in MOPC 315. The restriction enzyme mapping suggested that many of the MOPC 315 clones were members of a population of newly acquired IAP genes with a distinct set of conserved restriction enzyme sites. To explore this possibility further, unique cellular sequences flanking the IAP proviruses were isolated for four clones from MOPC 315 and four clones from MOPC 104E. Clones were selected that had the maximum number of conserved restriction enzyme sites, except for those from MOPC 104E, which all lacked the 1.6-kb *MspI* fragment. With the exception of clone 57 (104E), the flanking sequence probe was isolated from an *EcoRI* fragment that extended from the single *EcoRI* site in the IAP gene into the cellular DNA on the 3' side. Since both gene libraries were created by partial *EcoRI* digestion, this assured that the flanking sequences were linked to the IAP gene in the tumor DNA. The positions of these probes are shown in Fig. 4.

If a particular IAP gene is endogenous to the mouse genome, the flanking sequence probes should detect the same band in restriction digests of BALB/c and tumor DNA. Based on this analysis, five of the IAP genes tested are endogenous proviruses (Fig. 5). All four IAP flanking sequence probes from the MOPC 104E clones hybridized to restriction fragments in BALB/c and tumor DNAs that were equivalent in size to those found in the cloned genes. These results are consistent with the observation that the amplified 1.6-kb *MspI* fragment was not found in any of the MOPC 104E clones. Clone 98 from MOPC 315 was also endogenous by these criteria.

If an IAP gene is newly acquired in the tumor, it should be present on a restriction fragment that is equivalent to the cloned IAP gene from the tumor DNA but different from the fragment in BALB/c DNA. At least two and probably three of the MOPC 315 IAP genes are newly acquired (Fig. 6). Clones 38 and 76 hybridize to fragments in MOPC 315 DNA that are not present in BALB/c or MOPC 104E DNAs and

FIG. 4. Restriction enzyme maps of eight type IIB IAP genes, indicating the fragments used as probes for the cellular flanking sequences. Symbols: E, *EcoRI*; B, *BamHI*; S, *SstI*; H, *HindIII*; P, *PstI*; X, *XbaI*; Pv, *PvuII*.

FIG. 5. Endogenous IAP genes from the MOPC 104E library. Southern blots of BALB/c, MOPC 315, and MOPC 104E DNAs were hybridized with the cellular flanking sequence probes from clones 57 (a) and L72 (b). DNAs were digested with *BamHI*. Similar data were obtained with probes from clones 98 (MOPC 104E), L74, and 98 (MOPC 315) (not shown). The band of hybridization corresponds in size to that expected from the cloned gene in each case.

that are equivalent in size to the fragments in the IAP clones. The hybridization of the clone 179 flanking sequence probe was much more complex. At least 13 bands of varying intensity were evident in both BALB/c and tumor DNAs, indicating that the DNA sequence was repeated several places in the mouse genome. However, in MOPC 315 DNA, there is a band at 11 kb that is not present in either BALB/c or MOPC 104E DNA, and this fragment size is consistent with that of the original clone. Further experiments with a unique sequence probe from clone 179 have shown that the 11-kb band is found only in MOPC 315, whereas the 9-kb band represents the endogenous target sequence (data not shown). In summary, three out of four of the MOPC 315 IAP clones contain newly acquired proviruses, whereas all four genes isolated from MOPC 104E were endogenous.

DISCUSSION

Studies of the organization and activation of IAP genes have been hindered by the nearly 1,000 endogenous proviruses in the mouse genome. The development of a type II IAP gene probe has allowed us to focus on a specific subset of IAP sequences which are abundantly expressed in several plasmacytoma lines. Most significantly, a particular type II IAP sequence variant with a 2.8-kb internal *EcoRI-HindIII* fragment was found to be amplified in MOPC 315. Analyses of both total MOPC 315 DNA and isolated clones demonstrated that the amplified genes are a very homogeneous population with many conserved restriction enzyme sites. On the other hand, the endogenous IAP genes, represented by the MOPC 104E clones, are quite heterogeneous with

FIG. 6. Newly acquired IAP genes in MOPC 315. Southern blots of *Eco*RI-digested DNAs were hybridized with the indicated cellular flanking sequence probes from clones 76 (a), 38 (b), and 179 (c). A band of hybridization corresponding in size to the cloned IAP gene was found in MOPC 315 DNA but not in BALB/c DNA for each probe.

respect to the same set of restriction enzyme sites. All of these results are consistent with the idea that only one of the 20 to 30 type IIB IAP genes has been activated in the MOPC 315 tumor line. This may be due to the position of the particular provirus in an active chromosomal domain. These genes would then be amplified through reverse transcriptase in the intracellular virions and subsequent integration. However, it is important to consider two additional observations. One is that the same IAP gene is not activated in all plasmacytoma lines (Fig. 2). The second is that there are a small number of transcripts from the more abundant type I genes in all tumors. Like the type IIB genes, the activation of other IAP genes may be due to their presence in actively transcribed chromosomal domains. However, it is also possible that the type I genes are activated by an entirely different mechanism, such as a general activation of transcriptional units in tumor DNA with a subsequent low level of transcription from the nearly 1,000 type I genes.

Since IAPs are continuously expressed in plasmacytomas, the three MOPC 315-specific IAP genes could have integrated at any time during passage of the tumor over the 15 years since it was induced (16). Based on the relative intensity of the novel restriction fragments that contain the newly acquired provirus and the endogenous cellular target sequence, it is possible to deduce whether the tumor is clonal with respect to proviral insertion. If the integration occurred at the onset of plasmacytoma formation, the tumor DNA should contain the provirus in at least one of the two or more homologous chromosomes in the often polyploid tumor cells. However, if reintegration is occurring frequently in many different cells during propagation of the tumor, only a portion of the cells would contain the newly acquired provirus at that locus. Consequently, the hybridization of

the flanking sequence probe to the tumor-specific restriction fragment would be significantly weaker than to the germ line band. Based on these considerations, the MOPC 315 tumor line is more or less clonal for all three newly acquired IAP genes. For clone 76, the tumor-specific band is more intense than the germ line band, indicating that all of the cells probably have a provirus in at least one chromosome. In clones 38 and 179, the tumor-specific band is approximately one-fourth the intensity of the germ line band. This could be due to there being only a fraction of tumor cells containing the provirus or to proviral integration into one of the chromosomes of a near-tetraploid cell. An alternative explanation is that the clone 37 flanking sequence hybridized to repetitive sequence elements in BALB/c DNA, only one of which is modified by proviral insertion.

These studies demonstrate that IAP genes can be a significant source of genetic instability to tumor cells in which they are expressed. Integration of IAP proviruses could serve to inactivate cellular genes by insertional mutagenesis or to increase the expression of cellular genes by promoter or enhancer insertion. Both of these possibilities have recently been found to occur in certain plasmacytoma and hybridoma lines. In XRP24 the *c-mos* oncogene has been activated by insertion of an IAP gene (13), whereas in one hybridoma cell line, a functional K immunoglobulin gene has been inactivated by IAP insertion into an intron (14). Furthermore, we have recently found that the clone 179 flanking sequence contains a cellular gene which is significantly enhanced in expression in MOPC 315, presumably by IAP integration (work in progress). Thus, IAP genes can serve as frequent modifiers of cellular gene expression in any cells in which they are expressed. Since IAPs are found in oocytes and early embryos of normal mice (3, 5, 6), IAP genes could play a significant role in generating mutations in the course of development and evolution.

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