Characterization of a Trimeric Complex Containing Oct-1, SNAPc, and DNA*

(Received for publication, March 3, 1997, and in revised form, April 23, 1997)

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The human small nuclear (sn) RNA promoters contain a proximal sequence element (PSE), which recruits the basal transcription factor SNAPc, and a distal sequence element characterized by an octamer sequence, which recruits the POU domain transcription factor Oct-1. The Oct-1 POU domain and SNAPc bind cooperatively to probes containing a PSE and an octamer sequence, and this effect contributes to efficient transcription in vitro. In vivo, however, Oct-1 regions outside of the POU domain can activate snRNA gene transcription. Here, we have examined whether the role of these regions is to contribute to cooperative binding with SNAPc. We find that they indeed improve cooperative binding, but most of the effect is nevertheless mediated by just the POU domain. This suggests that Oct-1 activates transcription of snRNA genes in at least two steps, recruitment of SNAPc mediated primarily by the POU domain, and a later step mediated by regions outside of the POU domain. We also show that a PSE-binding complex observed in nuclear extracts consists of Oct-1 and SNAPc. Although Oct-1 cannot bind effectively to the PSE probe on its own, in the complex it contacts DNA. Thus, in a nuclear extract, SNAPc can recruit Oct-1 to a probe to which Oct-1 cannot bind on its own.

Transcriptional activators are key regulators of RNA polymerase II transcription, but their mode of action is still poorly understood. Activators often consist of a DNA-binding domain, whose role is to target the activator to the correct promoter, and of activation domains, whose role is to enhance transcription (1). The activation domains may help recruit members of the basal transcription machinery to the promoter, enhance transcription elongation, or perhaps trigger modifications of the basal machinery that result in enhanced transcription initiation (2–6) (see Refs. 7 and 8, for reviews).

The RNA polymerase II and III snRNA* gene promoters both contain an essential proximal sequence element (PSE), which recruits the basal transcription factor SNAPc (also called PTF) (9–11), and a distal sequence element, which serves as a transcriptional enhancer and is characterized by the presence of an octamer sequence. The octamer constitutes a binding site for both the Oct-1 and Oct-2 POU domain transcription factors, but the distal sequence element is thought to recruit Oct-1. Indeed, like snRNA genes, Oct-1 is broadly expressed, whereas Oct-2 is a B cell-specific factor (see Ref. 12, for a review). Moreover, in vivo, the Oct-1 and Oct-2 activation domains display promoter specific activities; the Oct-1 activation domains preferentially activate snRNA promoters, whereas the Oct-2 activation domains preferentially activate transcription from mRNA promoters (13, 14). This differential activation results from differences in the mRNA and snRNA basal promoter elements, suggesting that the Oct-1 and Oct-2 activation domains interact differentially with promoter-specific basal transcription factors (13).

Both the Oct-1 and Oct-2 POU domains bind cooperatively with SNAPc/PTF to a probe containing a PSE and an octamer sequence, and at least in the case of the Oct-1 POU domain, this cooperative binding promotes increased levels of transcription in vitro (9, 15). The observation that in vivo, Oct-1 regions outside of the POU domain activate snRNA gene transcription, and do so much more efficiently than Oct-2 regions outside of the POU domain, suggests that the POU domain is not sufficient for transcription activation in vivo (16). How do Oct-1 regions outside of the POU domain contribute, then, to transcription activation?

Here we have tested whether Oct-1 regions outside of the POU DNA-binding domain play any role in cooperative binding with SNAPc to probes containing a PSE and an octamer sequence. We find that they do contribute to cooperative binding but most of the effect is mediated by the POU domain, suggesting that the Oct-1 activation domains play their primary role at a later step in the activation process. We also show that in crude nuclear extracts, a complex consisting of Oct-1 and SNAPc forms on a probe containing a PSE-binding site but lacking an octamer site. Formation of the complex is dependent on the ability of Oct-1 to bind DNA, and indeed Oct-1 contacts DNA in the complex. Thus, in the very complex mixture of proteins that constitutes a nuclear extract, SNAPc can recruit Oct-1 to a probe to which Oct-1 cannot bind on its own.

EXPERIMENTAL PROCEDURES

Constructs

Constructs for PCR Probes—The plasmids containing the H2B octamer site and human U6 PSE were previously described (15). The plasmid containing the H2B octamer site was described previously (17). The plasmids AD (also referred to in the text as mouse U6 PSE probe) and the mouse U6 PSE probe with the ABC mutation were generated by annealing two oligos, filling in with the Klenow fragment of DNA polymerase, cutting with BamHI and HindIII, and inserting into pUC118. This resulted in plasmids containing inserts with the sequences GGAATCCGCAACTACCCCTAATTATGTTCTCTTGGCTTCTCGAGGTGGAGCTTAAAG and GGAATCCGCAACTACCCCTAATTATGTTCTCTTGGCTTCTCGAGGTGGAGCTTAAAG for the AD plasmid and the plasmid containing the mouse U6 PSE with the ABC mutation were generated by annealing two oligos, filling in with the Klenow fragment of DNA polymerase, cutting with BamHI and HindIII, and inserting into pUC118. This resulted in plasmids containing inserts with the sequences GGAATCCGCAACTACCCCTAATTATGTTCTCTTGGCTTCTCGAGGTGGAGCTTAAAG and GGAATCCGCAACTACCCCTAATTATGTTCTCTTGGCTTCTCGAGGTGGAGCTTAAAG for the AD plasmid and the plasmid containing the mouse U6 PSE with the ABC mutation, respectively. The N7 plasmid has been previously described (18). Probes were generated by PCR amplification of these constructs using the universal sequencing primer end-labeled with [γ-32P]ATP and T4 polynucleotide kinase and the re-
verse sequencing primer. The probes AD-mutHD and AD-short were generated by PCR with the plasmid AD as a template and primers with the sequences TCACACAGGAAAAGCAGCTATGACATCGACCGAGAT-TGCG and AGCTCGTTAGCCGGGATCC, respectively, substituted for the reverse sequencing primer. All the probes were generated with the same primer and had the same final concentrations.

**Expression Constructs**—The pET11c.G-POU-1 and pET11c.G-Pit-1 POU constructs, which contain the Oct-1 POU and Pit-1 POU domains fused to the glutathione S-transferase (GST) gene, were previously described (19, 20). The constructs pET11c.H-Oct-1 and pET11c.H.P.1.1 were generated by PCR amplification of a plasmid containing the Oct-1 coding sequence and pHOct-1-IHIP/PoP (20), respectively, using oligonucleotides with the sequences GGAATTCCTATGATCAGTACATCACTCA-CCATCACAACATCCGACCAAGC and ACGCGGATCCACTCATGTCGCTTGGAGGC. The PCR products were cleaved with Ndel and BamHI and ligated into pET11c cleaved with Ndel and BamHI.

### Sources of Proteins

**SNAP**—The SNAP, used in these experiments was derived from a Mono Q peak fraction, which corresponds to the fourth step in the purification of SNAP, and is purified approximately 2,500-fold (10). The Mono Q peak fraction, which corresponds to the fourth step in the purification of SNAP, was applied to a Mono S 5/5 column (Pharmacia) and eluted with a salt gradient from 0 to 400 mM imidazole. Fractions containing octamer binding activity were pooled, dialyzed and applied to an OctQ buffer containing 100 mM KCl, 20 mM HEPES, pH 7.9, 100 mM NaCl, 0.5 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining. In all cases the proteins appeared to be greater than 90% pure. Protein concentrations were measured by the Bio-Rad protein assay (Bio-Rad).

Histidine-tagged proteins were produced by growing 1-liter cultures of E. coli BL21 (DE3) cells expressing histidine-tagged Oct-1 (H.Oct-1) or histidine tagged Oct-1.P.1 (H.Oct-1.P.1) as described above. The cells were lysed by sonication in OctQ buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% Tween 20, 5% glycerol, 0.5% mercaptoethanol, 0.5% phenylmethylsulfonyl fluoride, 1 mg/ml BSA, 1 mM benzamidine, 2 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride) at 4°C. The cell lysate was centrifuged at 40,000 × g for 30 min. Supernatant was collected and passed over a Mono Q 10/10 column (Pharmacia). The flow-through fractions were kept and dialyzed against buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.5 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining.

**Nuclear Extracts**—HeLa cell nuclear extracts were prepared as described (22).

### EMSA

The binding reactions were performed in a total volume of 20 μl containing final concentrations of 100 mM KCl, 20 mM HEPES, pH 7.9, 5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, 20 μg of fetal calf serum as a protein carrier, 2 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.4 μg each of poly(dI-dC) and pUC118. The amounts of SNAP, and Oct proteins added are indicated in the figure legends. The reactions were incubated at room temperature for 20 min before addition of 25,000 cpm (50–100 μg) of radiolabeled DNA probe followed by a 30-min incubation at room temperature. The reactions were electrophoresed through 5% nondenaturing polyacrylamide gels (acrylamide/bisacrylamide ratio, 39:1) in 1× TGE running buffer (50 mM Tris, pH 8.0, 400 mM NaCl, 100 mM Tris, pH 9.0, 40 mM of solution A (2 mM 1,10-phenanthroline, 0.45 mM CuCl₂, 50 mM Tris, pH 8.0, 40 mM of solution B (58 mM 3-3-amidopropionic acid). The cleavage reaction was allowed to proceed for 13 min and stopped by the addition of 40 mM of 2,9-dimethyl-1,10-phenanthroline. The gel was incubated for an additional 15 min at 4°C and 10 min at room temperature. The gel was subject to autoradiography and the bands corresponding to specific protein-DNA complexes, as well as free probe, were excised. Gel slices were eluted overnight in 0.4 ml of 0.1% SDS, 5 mM EDTA, 20 mM Tris, pH 8.0. Eluted DNA was extracted with phenol: chloroform (1:1), precipitated with ethanol, and analyzed on a 6% polyacrylamide (19:1), 8 μTBE sequencing gel. The gel was dried and autoradiographed.
FIG. 1. Oct-1 is more active than Oct-1 POU in recruiting SNAPc to the PSE. A, EMSA with a probe containing the H2B octamer motif and a human U6 PSE in the absence of proteins (lane 1) or with 5 μl of a SNAPc fraction (Mono Q fraction (10)) alone (lane 2) or 5 μl of the SNAPc.
chimeric Oct-1 protein referred to as I.P.1 in which the Oct-1 POU domain was swapped with the POU domain of the pituitary transcription factor Pit-1 (20). The Pit-1 POU domain, which is 50% identical to the Oct-1 POU domain, binds to the H2B octamer motif (20) but does not efficiently recruit SNAPc, to the PSE (15). Any effect of Oct-1 regions outside of the POU domain on recruitment of SNAPc, to the PSE should, therefore, be easily visualized with the I.P.1 protein. The effects of I.P.1 and the Pit-1 POU domain on SNAPc binding to the PSE are shown in Fig. 2A. The amounts of octamer binding activity in the different protein preparations in the absence of SNAPc were determined as described above for the Oct-1 and Oct-2 proteins (data not shown), and the quantitated data are shown in Fig. 2B. SNAPc, on its own bound poorly to the probe (Fig. 2A, lane 2), but SNAPc, together with increasing amounts of Oct-1 bound up to 40-fold more efficiently to the PSE (Fig. 2, A, lanes 2–11, and B). When Oct-1 was replaced by I.P.1, a greatly reduced but clearly detectable enhancement of SNAPc binding was observed (3.5-fold at a 1.P.1 concentration equal to the highest concentration of Oct-1: see Fig. 2, A, lanes 12–20, and B). This enhancement was larger than that observed with just the Pit-1 POU domain (a maximum of less than 2-fold, see Fig. 2, A, lanes 21–29, and B). As with the Oct-1 protein in Fig. 1, the enhancement of SNAPc binding by the I.P.1 protein may be larger than that shown in Fig. 2B, because a significant fraction of the I.P.1 protein was not full-length and may have been missing a domain important for efficient SNAPc recruitment. Together, these results indicate that although the Oct-1 POU domain is primarily responsible for the recruitment of SNAPc to the PSE, Oct-1 regions outside of the POU domain also contribute to the effect in this assay.

A PSE-binding Complex Present in Crude Nuclear Extracts Contains SNAPc, and Oct-1—To search for any putative PSE-binding factors other than SNAPc, we performed EMSAs with a probe carrying the mouse U6 PSE alone, a high affinity binding site for SNAPc, and nuclear extracts. As a control, we used a probe carrying six point mutations in the PSE that debilitate U6 snRNA transcription both in vivo and in vitro (Ref. 18, and data not shown). As shown in Fig. 3, lane 5, we observed, in addition to the SNAPc-DNA complex, a second complex of slower mobility that did not form with the mutant probe (lane 12). The migration of both complexes was retarded after incubation with polyclonal antibodies directed against the SNAP43 (10) or the SNAP45 (25) subunits of SNAPc, (data not shown), and the quantitated data are shown in Fig. 2A. SNAPc, on its own bound poorly to the probe (Fig. 2A, lane 2), but SNAPc, together with increasing amounts of Oct-1 bound up to 40-fold more efficiently to the PSE (Fig. 2, A, lanes 2–11, and B). When Oct-1 was replaced by I.P.1, a greatly reduced but clearly detectable enhancement of SNAPc binding was observed (3.5-fold at a 1.P.1 concentration equal to the highest concentration of Oct-1: see Fig. 2, A, lanes 12–20, and B). This enhancement was larger than that observed with just the Pit-1 POU domain (a maximum of less than 2-fold, see Fig. 2, A, lanes 21–29, and B). As with the Oct-1 protein in Fig. 1, the enhancement of SNAPc binding by the I.P.1 protein may be larger than that shown in Fig. 2B, because a significant fraction of the I.P.1 protein was not full-length and may have been missing a domain important for efficient SNAPc recruitment. Together, these results indicate that although the Oct-1 POU domain is primarily responsible for the recruitment of SNAPc to the PSE, Oct-1 regions outside of the POU domain also contribute to the effect in this assay.

The Complex Containing SNAPc, and Oct-1 Does Not Form with a DNA-binding Defective Mutant of Oct-1—The SNAPc-Oct-1 complex can assemble on a probe to which Oct-1 alone cannot bind. We therefore asked whether SNAPc, and Oct-1 coexist as a complex in the absence of DNA. Attempts to co-immunoprecipitate Oct-1 with an anti-SNAPc antibody and vice versa were unsuccessful, suggesting that this is not the case (data not shown). In another approach, we asked whether the complex could form with an Oct-1 POU domain containing a single alanine substituted for an arginine at position 49 of the POU domain (R49A (12)). This Oct-1 POU domain mutant does not bind to an octamer site efficiently but can assemble into a VP16-induced complex with VP16 and HCF (12). As shown in Fig. 4, addition of either a histidine-tagged Oct-1 protein or a GST-Oct-1 POU domain fusion protein retards the migration of the SNAPc-PSE complex (lanes 3 and 4), suggesting that both proteins can form a complex with SNAPc on a PSE probe. Indeed, these complexes can be supershifted with anti-Oct-1 antibodies (lanes 9 and 10). In contrast, addition of a GST-Oct-1 R49A mutant protein does not retard the complex (lanes 5 and 6) and nor render it reactive to anti-Oct-1 antibodies (lanes 11 and 12). Thus, an Oct-1 mutant defective for binding DNA cannot assemble with SNAPc, on the PSE probe.

Oct-1 Contacts DNA When Complexed with SNAPc, on the PSE Probe—The observation that a mutant Oct-1 defective for binding DNA could not assemble with SNAPc, on the PSE probe raises the possibility that Oct-1 interacts with DNA in the complex. To examine this possibility directly, we performed orthophenanthroline-Cu (OP-Cu) footprinting (23, 24) on protein-DNA complexes fractionated by EMSA. We used three probes, designated AD, N7, and H2B Octa. As shown in Fig. 5A, the AD and N7 probes both contain the mouse U6 PSE, but the flanking sequences are different. The H2B Octa probe contains the H2B octamer motif, a high affinity site for Oct-1. The three probes were incubated with SNAPc, Oct-1, or both, and the binding reactions were fractionated by EMSA. The gel was then exposed to OP-Cu and the DNA fragments corresponding to free probe, or to probe complexed with SNAPc, or probe complexed with SNAPc and Oct-1 were eluted from the gel and fractionated on a sequencing gel. The results are shown in Fig. 5B. DNA from the Oct-1/H2B Octa probe complex displayed a footprint over the octamer motif, as expected (lane 8). DNA from the SNAPc/AD probe and SNAPc/N7 probe complexes showed a clear footprint on the PSE (lanes 2 and 5). Significantly, on both the AD and N7 probes, DNA from the complexes containing SNAPc and Oct-1 displayed, in addition to the footprint on the PSE, a footprint higher up in the gel (lanes 3 and

fraction with increasing amounts of His-Oct-1 (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 ng in lanes 2–17), Oct-1 POU (0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, and 2.56 ng in lanes 12–20), Oct-2 (1.25, 2.5, 5, 10, 20, 40, 80, 160, and 320 pg in lanes 21–29), and Oct-2 POU (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 pg in lanes 30–38). The positions of the free probe and of the complexes containing the Oct-1 or Oct-2 POU domains (POU), Oct-2, His-Oct-1, SNAPc, together with Oct-1 or Oct-2 POU (SNAPc/POU), or SNAPc, together with His-Oct-1 or Oct-2 (SNAPc/Oct) are indicated. B. EMSA performed with a probe containing the H2B octamer motif and a human PSE in the absence of proteins (lane 1) or with increasing amounts of the various Oct proteins indicated above the lanes (lanes 2–37). The amounts of each Oct protein in the titrations were identical to those used in A. The positions of the complexes containing Oct-1 or Oct-2 POU (POU), Oct-2, and His-Oct-1 are indicated. C. graph showing the fold enhancement of SNAPc binding by the different Oct proteins. The y axis corresponds to the fold enhancement of SNAPc recruitment by the various Oct proteins as determined by dividing the amounts of SNAPc-Oct-1 (□), SNAPc-Oct-2 (○), SNAPc-Oct-1 POU (○), and SNAPc-Oct-2 POU (△) complexes obtained in panel A by the amounts of complexes formed with SNAPc alone (lane 2). The x axis corresponds to the amount of complexes formed by the different Oct proteins in the absence of SNAPc, in panel B. The quantitations were performed with a PhosphorImager.
as shown in Fig. 5A, the distance between this additional footprint and the PSE footprint varies on the two probes, but in each case the same sequence is protected. The footprint corresponds to an ATT sequence within a region, ATGATTACGAA, with limited similarity to an octamer motif in either orientation (see Fig. 5C). These results suggest that Oct-1 contacts DNA in the complex, and that this point of contact is remarkably flexible relative to the location of the PSE.

Oct-1 recognizes a specific DNA sequence in the SNAPc-Oct-1 complex with DNA—The footprinting results above suggested that although Oct-1 can contact DNA at various distances from the PSE in the SNAPc-Oct-1-DNA complex, the sequences recognized by Oct-1 are specific. To confirm this possibility, we tested the ability of Oct-1 to supershift a SNAPc-PSE complex formed on a probe containing a mutation within the ATT sequence contacted by Oct-1 (AD-mutHD probe, see Fig. 5A), or a truncated probe missing this sequence altogether (AD-Short probe, see Fig. 5A). As shown in Fig. 5D, Oct-1 could “supershift” the SNAPc-DNA complex on the AD and N7 probes, as before, but very weak complexes, or no

Fig. 2. Regions flanking the Oct-1 POU domain can recruit SNAPc to the PSE in the absence of the Oct-1 POU domain. A, EMSA performed with a probe containing the H2B octamer motif and a human PSE, and 5 µl of the SNAPc fraction alone (lane 2) or 5 µl of the SNAPc fraction with increasing amounts of Oct-1 (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 ng in lanes 3–20), and Pit-1 POU (0.025, 0.050, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 ng in lanes 21–29) proteins. The positions of the free probe and of the complexes containing the Pit-1 POU domain, Oct-1, or Oct-1.P.1, SNAPc, and the Pit-1 POU domain (SNAPc/Pit-1 POU) or SNAPc, alone, and SNAPc, and Oct-1 (SNAPc/Oct-1) or SNAPc, and 1.P.1 (SNAPc/1.P.1) are indicated. Note that in lanes 19 and 20, there is a reduction in the SNAPc-PSE binary complex, indicating that the probe was limiting at these high concentrations of Oct-1.P.1. B, graph showing the fold enhancement of SNAPc binding by the different Oct proteins. An EMSA was performed with a probe containing the H2B octamer motif and a human U6 PSE, and the same amounts of the different Oct proteins used in panel A but no SNAPc. The amounts of Oct complexes formed under these conditions are on the x axis. The y axis corresponds to the fold enhancement of SNAPc recruitment by the various Oct proteins as determined by dividing the amounts of SNAPc/Oct-1 (∆), SNAPc/Oct-1.P.1 (○), and SNAPc/Pit-1 POU (□) complexes obtained in panel A by the amount of complexes formed with SNAPc alone (lane 2). The quantitations were performed with a PhosphorImager.
retarded complexes, were observed with the probe mutated within the ATT sequence (lanes 11–15), or the truncated probe (lanes 16–20), respectively. These results show that Oct-1 requires sequence-specific interactions with the DNA to form the SNAPc-Oct-1-DNA complex.

**DISCUSSION**

The snRNA promoters contain an enhancer, the distal sequence element, which is nearly always characterized by the presence of an octamer sequence as well as, in some cases, an Sp1-binding site (28). This contrasts with the enhancers of mRNA promoters, which differ from one promoter to the next in a sequence-specific manner, and that this contact is required for formation of the complex. Thus, as we had observed previ-
ously, cooperative binding of SNAPc and Oct-1 to DNA requires Oct-1-DNA contacts. Interestingly, the location of these contacts relative to the location of the PSE is flexible and changes on different probes. This is consistent with the observation that neither the distance between the octamer sequence and the PSE (9) nor the orientation of the octamer (15), are critical for cooperative binding.

The POU domain consists of two helix-turn-helix-containing DNA-binding structures, the POU homeodomain (POUH) and the POU-specific domain (POUS), joined together by a flexible linker (30–34). Cooperative binding of the Oct-1 POU domain and SNAPc can be disrupted by a single amino acid change within the POUc domain, which maps to the surface of helix 1 away from the DNA and has no effect on DNA binding (15). This suggests that the POUc domain is involved in direct protein-protein interactions with SNAPc, and that its position

![Image](image_url)
may, therefore, be fixed relative to SNAPc and the PSE. We show here that cooperative binding can also be disrupted by a single amino acid mutation within the POUH domain that maps to the surface of helix 3 pointing toward the DNA and that affects DNA binding (12). This suggests that the POUH domain also contacts the DNA in the complex. How, then, can there be so much flexibility in the spacing between the PSE and the sequences contacted by Oct-1 (12)? Perhaps the location of SNAPc and the PSE. Indeed, the sequence ATT (or AAT on the other strand) constitutes part of the AAAT sequence recognized by the POUH domain on a histone H2B-octamer site (33). Thus, perhaps on different probes, the relative locations of the Oct-1 POU and POUH domains changes, the first being dictated mainly by the location of SNAPc, and the second by the local DNA sequence. Alternatively, SNAPc may itself be flexible, allowing different positionings of Oct-1 on the DNA while maintaining protein-protein contacts.

Acknowledgments—We thank M. A. Cleary, W. Herr, V. Mittal, and M. Tanaka for reagents and discussions. We also thank W. Herr and V. Mittal for comments on the manuscript, and M. Ockler, J. Duffy, and P. Renna for artwork and photography.

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