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STRUCTURE AND FUNCTION OF THE MHC CLASS II GENE TRANSCRIPTION FACTOR RFX

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RFX is a transcription factor essential for expression of MHC class II genes. cDNA clones encoding human and mouse RFX have been cloned and characterized. Human and mouse RFX show strong conservation (>90%) particularly in the DNA binding and dimerization domains (100%). The DNA binding and dimerization domains are novel, and are both structurally and functionally independent of each other. The binding site in the DRA gene (the cis-acting X box of class II gene promoters) has been defined precisely using mutated oligonucleotides. When bound, RFX interacts with a protein binding to a TRE like sequence situated immediately adjacent to the X box. Finally, cotransfection of an RFX expression plasmid and a reporter gene shows that RFX activates transcription from class II gene promoters.

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THE HLA CLASS II TRANSCRIPTION FACTOR RF-X IS PHOSPHORYLATED

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In normal B cells RF-X binds to the X box of HLA class II promoters whereas in CID B cells (patients suffering from class-II deficient combined immunodeficiency) no binding is detected. cDNA cloning has shown that in at least one patient this defect is not due to a mutation in RF-X.

We are therefore studying possible post-translational modifications such as phosphorylation that might affect binding of RF-X. RF-X is phosphorylated in both normal and CID B cells. We are currently examining whether the state of phosphorylation influences binding activity, and whether there is a difference in the pattern of phosphorylation in CID cells.

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TRANS-DOMINANT EXTINCTION OF HLA CLASS II GENE EXPRESSION BY ANALOGUES OF THE REGULATORY FACTOR RF-X.

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The expression of the products of MHC genes controls the immune response and their expression is highly regulated. Several protein factors bind to class II promoters and one such factors, RF-X, was shown to be essential for HLA class II gene expression. Following the cloning of the RF-X cDNA, the structure of this factor was determined and its DNA-binding domain was mapped. We have explored the possibility that expression of an excess of a truncated form of RF-X, inactive as a transcription factor would compete for the binding on HLA class II promoters with the normal form and lead to dominant suppression of class II gene expression.
Control experiments showed that the binding of RF-X to DNA in vitro could indeed be inhibited by these truncated forms of RF-X. We then constructed expression plasmids that allow the synthesis of various portions of RF-X, including the DNA-binding domain, linked to a nuclear targeting sequence. These were transfected into a human fibroblast cell line and the effect of these truncated forms of RF-X on HLA class II gene expression was studied. Indeed, in these stable transfectants, the level of HLA class II molecules expressed (in response to gamma interferon) was drastically reduced. The various controls will be discussed. This experiment shows that transdominant repression of MHC class II gene expression can be achieved by a polypeptide that competes with RF-X for its binding on HLA class II promoters.

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DOWN-REGULATION OF MHC CLASS II GENE EXPRESSION BY ANTISENSE INHIBITION OF REGULATORY FACTOR RF-X

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**The regulation of MHC class II gene expression is under tight control and directly influences T cell immune responses.
We here explore the possibility of achieving experimental downregulation of MHC class II expression through antisense inhibition of one of the regulatory factors essential to their transcription, RF-X.
RF-X antisense oligonucleotides were evaluated for their inhibitory effect on in vitro RF-X protein synthesis. In vivo treatment of human monocytic cells with selected antisense oligomers was demonstrated to largely inhibit gamma interferon induction of MHC class II molecules at the cell surface.
Functional consequences of this down-regulation on antigen specific, MHC restricted T cell stimulation were then measured as a new mean toward immunomodulation.**

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MODULATION OF TRANSCRIPTIONAL FACTOR ACTIVITY IN HUMAN MONOCYTIC CELLS. Matthew J. Fenton[1], Christophe Baumberger, and Jean-Michel Dayer. Dept. of Medicine, Boston University Medical Center[1], Boston, MA 02118, USA and Division of Immunology and Allergy, Dept. of Medicine, Hôpital cantonal universitaire, 1211 Geneve 4, Switzerland.

Interleukin 1 (IL-1) is a pluripotent proinflammatory cytokine that is expressed by numerous cell types, including activated cells of the macrophage/monocyte lineage. Two unique IL-1 genes (IL-1α and β) have been identified in both human and murine cells. Expression of the IL-1β gene appears to require the presence of the novel transcriptional regulatory factor NFIL-1βA. We have used electrophoretic mobility shift assays (EMSA) to examine the expression of several NFIL-1βA isoforms in nuclear extracts prepared from U937 human histiocytic lymphoma cells stimulated with PMA or LPS, and stimulated cells that have been previously exposed to gamma interferon (γ-IFN). We have observed changes in isoform-specific DNA-binding activities within 15 minutes following activation. In particular, one isoform is transiently expressed in cells stimulated with PMA, but is not expressed in LPS-stimulated cells. Since pretreatment of the cells with γ-IFN prior to activation has been previously shown to augment IL-1 gene expression, we have also examined the ability of γ-IFN to regulate the NFIL-1βA isoform levels. Together, these data suggest that NFIL-1βA levels can be differentially regulated by distinct macrophage activating factors.

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In vitro TRANSCRIPTION FROM THE IO PROMOTER OF THE VITELLOGENEIN GENE A1 FROM *X. laevis*.

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The 5'-end region of the *Xenopus laevis* vitellogenin gene A1 contains two estrogen-responsive transcription initiation sites termed i and io, 1.8 Kb apart. In estrogen-induced hepatocytes, the upstream site io is much weaker then the dowstream i site which is homologous to the one used by the other members of the *Xenopus* vitellogenein gene family. Interestingly, in *in vitro* transcription assays using HeLa cell nuclear extracts the two sites have a similar strength. There are three Sp1 binding sites upstream of the io, wich can bind purified Sp1. However, in the extracts used in the *in vitro* transcription experiments there is another factor able to recognize this promoter. Analysis of competition experiments suggests an inhibitory effect of this factor on the activity of the io promoter. The charactherization and function of this factor are on the way to be determined.

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TISSUE SPECIFIC EXPRESSION OF THE GRP 1.8 GENE IS MEDIATED BY DIFFERENT REGULATORY ELEMENTS

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GRP 1.8, a glycine-rich cell wall protein of bean (*Phaseolus vulgaris*), is expressed specifically in developing vascular tissues. Gene constructs consisting of GRP 1.8 promoter fragments of different length were translationally fused to the coding region of the GUS reporter gene and resulted in vascular specific gene expression in stems.
The promoter sequence from -205 to 0 (-205) was sufficent to give specific expression. Shorter promoter fragments showed expression in the epidermis in addition to the normal expression pattern , and the -121 construct had completely lost specificity (expression in most cell types). The -94 construct conferred only little expression in all organs except root tips, where expression was still high. A -76 promoter fragment was inactive.
These findings suggest that at least three regulatory elements are involved in GRP 1.8 expression.
In gel retardation assays and DNase I footprintings we found a tobacco nuclear factor binding to the GRP 1.8 promoter around position -190.
In future experiments we will determine the exact binding site with synthetic oligonucleotides in gel retardation assays.

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ANALYSIS OF THE TENASCIN PROMOTER IN NORMAL AND TRANSFORMED CHICKEN EMBRYO FIBROBLASTS (CEF).

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Tenascin (TN) is an extracellular matrix protein transiently expressed in many developing organs and the stroma of tumors. To investigate the mechanisms of Tn gene regulation, we isolated the Tn promoter from a chicken genomic library . Sequence analysis showed a TATA box, AP1, Oct, and NF1 binding site within 300 bp of the transcription start point. Six CCAAT boxes are found further upstream between 1.8 and 4.0 kb. To study the known induction of Tn by FCS and TGF-beta in cultured CEFs, we performed transient transfection assays using constructs containing the Tn promoter and the firefly luciferase as the reporter gene. We compared promoter activity in normal and temperature-sensitive v-src expressing (RSV infected) CEFs. We noticed a 20 fold higher activity of Tn promoter in transformed CEFs grown at the permissive temperature.

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PROMOTER ANALYSIS AND GENE RESCUE OF THE *DROSOPHILA GOOSEBERRY* LOCUS

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Embryonic development in *Drosophila* depends on the three hierarchically acting classes of segmentation genes, the gap, the pair-rule and the segment-polarity genes. These genes act to progressively subdivide the embryo into a series of metameric units. During the last step of this process, the segment-polarity genes specify the orientation of the segments. The *gooseberry* locus belongs to the class of segment-polarity genes and consists of two transcription units which encode proteins that share two homologous sequences, the homeo and the paired domain. Furthermore, the 5' ends of both genes face each other and are separated by about 10kb. Both genes are expressed in an overlapping set of cells, although at different periods of embryogenesis. To investigate their function and possible interactions with each other, we will present results from (i) a promoter analysis of the two genes, performed both on wild type and mutant backgrounds, and (ii) gene rescue experiments.

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ASTROCYTES , NEURO-AND GLIOBLASTOMA CELLS EXPRESS NOVEL OCTAMER-DNA BINDING PROTEINS

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The "octamer" sequence, ATGCAAAT (ATTTGCAT), is a key element for transcriptional regulation for a number of genes in various cell types. The ubiquitous protein Oct-1 controls general octamer site-dependent transcription, while the Oct-2 protein contributes to B-cell specific gene expression in lymphocytes. We have analyzed the octamer-binding proteins in cells of the nervous system. Using the bandshift assay, at least six novel octamer binding proteins were detected in nuclear extracts of cultured mouse astrocytes. These proteins are differentially expressed in human gliablastoma and neuroblastoma cell lines. We found that all N-Oct-factors were distinct from the ubiquitous Oct-1 and the lymphoid Oct-2 proteins (Nucl.Acids Res. 1990, p. 5495 ff.). From cloned Oct-proteins it is known that they contain a homologous DNA-binding domain, called POU-homeodomain. Currently, we use oligo primers derived from highly conserved aminoacid residues to amplify by PCR cDNAs of the POU-homeodomains of N-Oct proteins. These will serve as probes for screening for full length cDNAs. The cloned cDNAs of N-Oct proteins should enable us to elucidate their function in regulating gene expression in the nervous system.

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MURINE INTERFERON REGULATORY FACTOR 1 (IRF-1) IS ACTIVE IN SACCHAROMYCES CEREVISIAE

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Virus induction of the IFN-ß promoter is mediated by 4 sequence elements: The negatively acting NRDI, PRDI and PRDIII, which bind the activator IRF-1 or the repressor IRF-2, and PRDII, which binds NF-κB. Virus induces and/or activates IRF-1 and activates NF-κB (Taniguchi et al., Maniatis et al., Hiscott et al.). $(GAAAGT)_4$ preceding a minimal promoter mediates virus induction and response to IRF-1 by mimicking dimeric PRDI (Näf et al.).
We placed $(GAAAGT)_4$ upstream of a minimal yeast promoter/ß-galactosidase construct. Yeast containing this reporter and an inducible IRF-1 expression plasmid expressed ß-galactosidase upon IRF-1 induction. IRF-1 has the same sequence specificity in yeast as in mammalian cells. These results show that IRF-1 interacts with the transcriptional machinery of yeast, and suggest it is the immediate activator of PRDI and no specific modifications are required for its activity.

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FUNCTIONAL ASPECTS OF THE HOMEODOMAIN STRUCTURE

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The homeodomain (HD) is a highly conserved 60 amino acid peptide domain present in a large number of transcription factors that are involved in regulating the spatially and temporally coordinated expression of genes during developmental processes. The HD portion is required for DNA binding of these factors to distinct DNA target sites and might therefore contribute to the target site selection of HD proteins.
To address the question of how HD proteins recognize their target sites, we initiated *in vitro* DNA binding studies using isolated HD peptides and synthetic binding sites. We found that isolated HD peptides bind specific binding sites with high affinity and make contact with DNA over an extended segment covering a major and the adjacent minor groove. Studies with mutant HD peptides allowed the localization of amino terminal residues to the minor groove and residues present along helix III (the recognition helix of HDs helix-turn-helix motif) to the major groove. We are presently persuing different experimental procedures to find out which regions (in addition to the recognition helix) contribute to the *in vitro* target site specificity of HD proteins. Our functional studies will be discussed with respect to results obtained from structural studies of an *Antp* HD-DNA complex (Otting et al., EMBO J. 9, 3085-3092, 1990).

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AMINO ACID RESIDUES AFFECTING THE DNA-PROTEIN INTERACTION OF THE *FUSHI TARAZU* HOMEODOMAIN OF *DROSOPHILA MELANOGASTER*

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The homeodomain (HD) is a distinct structural motif of 60 amino acids. It consists of four α-helices and mediates sequence-specific DNA binding. In order to disclose the critical residues of the *fushi tarazu (ftz)* HD for its activity, we exchanged the middle part of the HD with the corresponding sequences of the *Sex combs reduced (Scr)* and the *muscle segment homeobox (msh)* HDs. With *ftz* rescue tests in flies and transfection assays in cultured cells, we have found that the *Scr* chimera retains *ftz* wild type activity whereas the *msh* chimera does not. Analyses with a series of backmutations of the *msh* chimera have revealed that the stretch of RRR (28-30), particularly R28, is crucial for the full activity in both flies and tissue culture cells. The R-stretch located at the amino terminus of helix 2 is among the invariable parts of the *Antennapedia (Antp)* class of HDs. In addition, we have found that Y25 and R31, which are highly conserved in all classes of HDs, are also very important for *ftz* activity. The three-dimensional structure of the *Antp* HD-DNA complex suggests direct contacts by these residues to the sugar-phosphate backbone of the target DNA. Similar contacts are known to be required for the correct positioning of the recognition helix in the major groove in the prokaryotic repressor-DNA complexes.

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CHARACTERIZATION OF A *XENOPUS LAEVIS* VITELLOGENIN PROMOTER IN *SACCHAROMYCES CEREVISIAE*

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Transcription from the vitellogenin promoter is strictly regulated by estrogen through its hormone receptor. The ligand bound estrogen receptor binds to a specific palindromic DNA sequence, and activates transcription. Little is known as to how this activation through the estrogen receptor works. We established a model system in yeast to analyse the mechanism of transcription regulation. The DNA coding for the *Xenopus* estrogen receptor was functionally expressed in yeast under the control of the yeast *GAL1* promoter. The vitellogenin B1 promoter fused to the bacterial *CAT* reporter gene was subcloned into a yeast vector. Different promoter deletion mutants were analysed *in vivo* with respect to transcription activation through the estrogen receptor. This yeast model system promises to be useful for further *in vitro* studies of the silent and activated vitellogenin promoter structure.

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ROLE OF CTF/NF1 IN THE REGULATION OF GENETIC EXPRESSION BY TRANSFORMING GROWTH FACTOR-ß1.

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Previous data have indicated that promoters containing binding sites for the transcription activator CTF/NF1 could be regulated by the cell growth and differentiation factor TGF-ß1. Here we have investigated wether CTF-1 and CTF-2, two members of the CTF/NF1 family, are targets for the TGF-ß1 regulation of gene expression. Our results indicate that the proline-rich transcriptional activation domain of CTF-1, when fused to the DNA binding domain of GAL4, confers TGF-ß1 regulation to a GAL4 responsive promoter in mouse fibroblasts. In contrast, a similar fusion to the activation domain of CTF-2 is not regulated by TGF-ß1 and thus activates transcription constitutively. These results suggest that specific members of the CTF/NF1 family of transcription factors are differentially regulated by TGF-ß1, probably at the posttranslational level. A possible regulatory role of CTF/NF1 species phosphorylation state will be discussed.

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SYNERGISTIC TRANSCRIPTIONAL ACTIVATION BY CTF/NF-I AND THE ESTROGEN RECEPTOR INVOLVES A LIMITING TARGET FACTOR.

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We have analyzed the mechanisms of synergistic transcriptional activation by CTF/NF-I (CTF-1) and the estrogen receptor (ER). Using GAL4-CTF-1 chimeric activators, we find that the proline rich activation domain of CTF-1 mediates synergism with the ER. Mutations in CTF-1 activation domain correspondingly affect transcriptional activation and synergism with the ER. Cooperative DNA binding by the two activators does not occur in vitro, but in vivo squelching experiments suggest that CTF-1 and ER use a common limiting target factor to activate transcription. Furthermore, both activators functioning synergistically are much more resistent to squelching than either one alone. Thus transcriptional synergism between CTF-1 and the ER may result from the stronger interactions of both activators with a limiting target factor, rather than from a direct cooperative DNA binding mechanism.

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TRANSACTIVATION BY THE GLUCOCORTICOID RECEPTOR

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We have studied the transactivation behaviour of wild type or mutated glucocorticoid receptor (GR) by expressing GR-cDNAs along with appropriate reporter genes and other non-ubiquitous trans-activators in mammalian cells. (1) We found that in presence of relatively small amounts of GR, the action of a distinct exogenous trans-activator such as the lymphoid-specific Oct2A is significantly impaired. Control experiments demonstrate that the interference is not caused by competitive target DNA binding. We speculate that the inhibition of Oct2A dependent trans-stimulation is due to titration of an accessory rate-limiting factor. Not surprisingly the interference is non-reciprocal (i.e. even a large excess of Oct2A does not inhibit GR-dependent trans-stimulation in non-lymphoid cells). (2) In a second set of experiments we have analyzed the effect of substitutions by every possible aminoacid at the position of the evolutionary ultra-conserved Cys500 residue. This position is conserved among all the members of the steadily growing nuclear receptors super-family, and had been erroneously proposed by several authors to be involved in metal chelation. The results indicate that several aminoacids can functionally substitute Cys500 under our assay conditions, including Gly which had been described by others as non-permissive. Interestingly, we found that one particular substitution (Glu 500) permits specific DNA binding but yields a null-transactivation mutant.

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STUDYING TRANSACTIVATOR-TARGET INTERACTIONS

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We described earlier that a particular truncated glucocorticoid receptor (GR N-556, lacking the hormone binding domain) can efficiently stimulate (about 1000-fold) transcription upon activation of promoters or enhancers consisting of closely spaced, idealized GR binding sites (GREs, Severne et al. 1988; *EMBO J.* 7, 2503-). Intact GR (GR N-795), stimulates these artificial GRE clusters only very inefficiently (50 fold). Both GR forms stimulate equally well a natural GRE cluster such as the one contained in the MMTV promoter. Systematic studies on artificial promoters in which the idealized GREs have been separated by different distances (from 24 to 96 bp, center-to-center) demonstrate that the intact GR displays a distinct cooperative stimulation, which reaches a maximum when the GREs are spaced by 60-70 bp. This distance is consistent with the idea that chromatin structure might be involved in the process of cooperativity and corresponds strikingly with the repeat length of strong viral enhancers. It is interesting to note that the 60-70 bp distance required for cooperativity maximum seems to be dictated by the presence of the hormone binding domain, which so far has never been suggested to play a role in the target recognition process. Regarding a second type of inter-domain cooperation, we also have identified a mutant in the DNA binding region (Lys 461->Tyr) , which is permissive for transactivation when tested as small GR fragment 407-556 but fails to increase its transactivation (as do the wild type and all other mutants) when the GR fragment is extended to include the entire N-terminus.

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CLONING OF cDNA SEQUENCES CODING FOR A STEROID RECEPTOR HOMOLOGUE IN CHIRONOMUS (DIPTERA)

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We have cloned the cDNA sequence coding for a Chironomus tentans protein (536 amino acids) which exhibits a good amino acid sequence homology to various members of the steroid and thyroid hormone receptor superfamily. This homology holds true not only for the zinc finger-containing DNA binding domain but also for the ligand binding domain, and particularly for the highly conserved regions within that domain. The Chironomus protein shows a far better sequence homology to the Drosophila ecdysone receptor (M. Koelle and D.S. Hogness, personal communication) than to E75A, svp and 2C, other members of the Drosophila steroid and thyroid hormone receptor superfamily. Therefore, we speculate that the cloned sequence codes for a Chironomus ecdysone receptor. The corresponding gene is located on chromosome 2, region 17C, as determined by *in situ* hybridization to salivary gland polytene chromosomes. On Northern blots, the cloned cDNA hybridizes to an approximately 4.3 kb polyadenylated RNA. Its expression seems to be developmentally regulated and correlates with changes in the ecdysone titer.

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CLONING AND EXPRESSION OF A XENOPUS cDNA HOMOLOGOUS TO THE CTF/NF-I GENE FAMILY.

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In vitro studies have suggested that tissue and sex specific expression of the *Xenopus Laevis* B1 vitellogenin gene is contributed to by analogues of the CTF/NF-I transcription activators.

We have therefore cloned 4 distinct cDNAs, from a Xenopus XTC kidney cell library, coding for homologues of CTF/NF-I proteins. The sequence of one of them, xCTF, encodes a highly conserved N-terminal part as compared to human CTF-1 which mediates DNA binding and oligomerization. However, the C-terminal part of the deduced polypeptide diverges from human CTF-1 but is more related to the hamster NF1/X subtype. The expression and functional properties of this clone will be presented.

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A NEW VECTOR FOR *IN VITRO* EXPRESSION OF DNA-BINDING MOTIFS

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The first and by far the best characterized structural motif for a DNA-binding domain is the helix-turn-helix motif, found in prokaryotic activator and repressor proteins as well as in the homeoboxes and thus in octamer-binding factors, too. It seems that this structural motif has been conserved through evolution. In fact, structural and sequence homologies can be found in prokaryotic transcription regulators and the oct-2 protein for instance.

The cloning of cDNA's encoding DNA-binding proteins and/or the expression in E. coli may therefore give rise to problems. The proteins might be toxic for the bacteria because of their DNA-binding activity.

The vector pBS2N SK (-) was constructed on the basis of Bluescript [pBS SK (-)] vector, with all its properties, for the rapid analysis of cDNA clones encoding DNA-binding proteins. Even if the cDNA is not a full length clone, it can be assayed for the presence of a funtional DNA-binding domain.
This vector was tested using the cDNA of the complete POU domain of a new mouse oct-2 variant.

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IDENTIFICATION OF PROTEIN BINDING SITES IN GENOMIC DNA BY TWO-DIMENSIONAL GEL ELECTROPHORESIS.

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The isolation of the target sequence of a DNA binding protein is an important step to characterize its functional role. However, it is often impossible to confidently predict protein binding sites by the mere inspection of a DNA sequence. Using available techniques (footprinting,gel shift assay)systematic search for protein binding sites can only be performed on cloned DNA fragments. We describe a simple two dimensional electrophoresis procedure to identify recognition sites of DNA binding proteins within complex DNA molecules. Using this approach, we have mapped E.coli IHF (Integration Host Factor) binding sites within phage lambda (48kb)DNA. We are also able to visualize IHF binding sites in E.coli chromosomal DNA (4700 kb). The combination of 2 D electrophoresis with PCR offers a powerful way to isolate rare protein binding sites, and allows the cloning of a collection of target sequences for DNA binding proteins in complex genomes.Individual binding sites can be amplified by PCR directly from the dried gel after addition of synthetic linkers and subsequently cloned in a suitable vector.

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CHARACTERIZATION OF A LYMPHOCYTE SPECIFIC DNA-BINDING PROTEIN

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We have previously described a nuclear protein complex which is constitutively present in a rodent T cell line (PC60) and bindings to the SV40 enhancer TC-II motif. This complex has a similar DNA sequence specificity as NF-κB, but displays opposite sensitivity to nucleotides (E.Espel et al., 1990, EMBO J. 9:929). More recent results strongly suggest that this PC60 protein is not related to NF-κB.
1/ Competition of binding to a TC-II probe with TC-II oligonucleotides containing single point mutations revealed several differences in specificity between the PC60 protein and NF-κB. 2/ IkB does not inhibit DNA binding of the PC60 protein but blocks NF-κB from the same cells. 3/ Antibodies against human NF-κB/p50 do not affect bandshifts due to the human homologue of the PC60 protein.
4/ Trypsin digestion of the PC60 protein produces a DNA-binding fragment that gives rise to DNA-protein complexes migrating faster than those formed by NF-κB fragments resulting from the same digestion.
5/ The PC60 protein is present in the nuclei of all B and T cell lines tested, in normal resting B and T lymphocytes and in some non-lymphoid hemopoietic cell lines. But it is undetectable in all non-hemopoietic cell lines screened, including fibroblasts, carcinomas, a hepatoma and pancreatic tumor cells. This distribution suggests that it could play a role in lymphocyte differentiation.

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SEQUENCES INVOLVED IN EFFICIENT TRANSCRIPTION OF THE C-MYC I GENE OF XENOPUS LAEVIS.

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Transcription of Xenopus laevis genes c-myc I & II is differentially regulated by two promoters P1 and P2. By microinjecting into Xenopus oocytes a series of deletion mutant constructs, linked to the reporter gene CAT, regions controlling transcription of Xenopus c-myc I gene were defined. Sequence between -158 and -28, relative to the P2 transcription initiation site, appeared to be involved in efficient transcription from both P1 and P2 promoters. The region identified as the essential promoter element contained three binding sites for SP1 and one for the transcription factor E2F which is known to be essential in the activation of adenovirus early promoters and enhancers, and also interacts with human c-myc promoter. We also find that the region -58 to -28 nucleotides, containing one binding site for SP1 and another for E2F, is most essential for transcription from the promoter P2.

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MYO D ACTIVATES XENOPUS SKELETAL ACTIN GENE TRANSCRIPTION BY INTERACTION WITH TWO BINDING SITES.

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Striated muscle actins in Xenopus laevis expressed in the myotome after the beginning of accumulation of Myo D transcripts. Myo D activates the transcription of some muscle specific genes. We show, by co-injection in Xenopus oocytes, that Myo D is also able to activate transcription from skeletal actin III promoter constructs. 153 bp of 5' sequence (-125 to +28) encompassing the first two CArG boxes are sufficient for the transactivation. By in vitro DNA-binding experiments with purified Myo D protein, we find two Myo D binding sites, one each flanking the transcription start site. These recognition sequences share only partial homology to the consensus.

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MODULATION OF XENOPUS ZINC FINGER PROTEIN TFIIIA-DEPENDENT TRANSCRIPTION ACTIVATION BY THIONEIN (APOMETALLOTHIONEIN)

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The rapid induction of thionein (apometallothionein) by many stimuli such as steroid hormones, cytokines and second messengers suggests an important but as yet undefined role in cellular activation processes. In view of the tendency of thionein to form strong complexes with zinc, we suggest that the effect of its enhanced biosynthesis is to reduce intracellular free zinc concentration and thus to modulate the actions of zinc-dependent proteins, most notably those of the zinc finger transcription factors. In testing this proposition, we have found by DNA binding and *in vitro* transcription assays that thionein indeed suppresses the activity of the vertebrate zinc finger transcription factor Sp1 but not of the zinc-independent homeobox-containing transcription factor Oct-1 (Zeng, J., Heuchel, R., Schaffner, W. and Kägi, J.H.R., submitted for publication). We have now also observed this inhibitory effect with the Xenopus zinc finger transcription factor TFIIIA. In the presence of thionein, binding of TFIIIA to both the 5S RNA gene and its product 5S RNA was abolished and its ability to initiate the 5S RNA gene transcription *in vitro* was lost. In contrast, the transcription of the TFIIIA-independent $tRNA_{arg}$ gene was not affected by thionein.

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PURIFICATION OF A TRANSCRIPTION FACTOR WHICH ENHANCES THE cAMP RESPONSE IN THE PIG uPA GENE PROMOTER

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Functional analysis of the promoter of the pig urokinase type plasminogen activator (uPA) gene showed that the region located 3.4 kb upstream of the cap site contained a cAMP inducible enhancer complex. This complex is comprised of two CRE consensus sequences (A+B) and a region (C) unrelated to the CRE consensus. The C region has specific DNA binding activity as shown by DNase I footprinting assays. It increases the cAMP response of the uPA gene by enhancing the effectiveness of the A and B regions. The C DNA-protein complex increases the binding of transcription factors to the CRE consensus sequences indicating cooperative protein binding. Gel retardation assays using nuclear extracts from LLC-PK1 cells indicate that at least two proteins bind the C region. Their molecular weights are between 35-45 KD and 80-100 KD as determined by UV-crosslinking and gel-elution and renaturation experiments. The purification and characterization of transcription factors binding the C domain of the pig uPA gene promoter will be discussed.

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Characterization of *cis*- and *trans*-acting Elements Mediating Induction of the Porcine uPA Gene During Cytoskeletal Reorganization

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uPA initiates an extracellular proteolytic cascade by converting plasminogen to plasmin which in turn can activate procollagenases. Proteolysis of extracellular structures plays a role in many normal and pathological cellular growth and migration processes. During these events uPA expression is often elevated and there are changes in the cytoskeleton. Previous work from this laboratory has indicated that cytoskeletal reorganization may directly induce uPA gene transcription and it does not require cAMP-PK or PK-C. In order to further investigate this signal transduction pathway we have identified the element in the porcine uPA gene promoter, with enhancer-like properties, that responds to cytoskeletal reorganization. We are also characterising the DNA-binding proteins involved.

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THE POLY(ADP-RIBOSE) PROTEIN SHUTTLE OF CHROMATIN

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Over the past five years, we have combined in vitro and in vivo approaches to determine the biological role and molecular mechanisms of the poly ADP-ribosylation system of chromatin. Our findings include: i) Two enzymes of the poly ADP-ribosylation system [poly(ADP- ribose)polymerase and -glycohydrolase] cooperate to shuttle histones H1, H2A, H2B, H3, H4 and protamine (but none of > 20 other proteins tested) off and onto the DNA template. Several enzymes tested can access histone-associated DNA only when the histone shuttle is operating. The shuttle mechanism is activated by DNA nicks both in in vitro and in vivo. The macromolecular species responsible for histone-binding is a homopolymer of ADP-ribose residues. The polymer-histone interactions are very strong (but noncovalent), reversible by DNA, hierarchical with regards to binding strengths (H1>H2A=H2B>H3>H4), specific with respect to the polymer structures involved, and self-adaptive to a specific shuttle target. - In vivo studies suggest an involvement of this shuttle mechanism in the unfolding of chromatin domains during DNA excision repair. (NF grant 3.161.088)

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A WAY TO CLONE *C.ELEGANS* TELOMERES

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Telomeres are specialized structures conferring stability to chromosomes by protecting them from degradation, recombination and by allowing complete replication of the ends. In the nematode *C.elegans*, they are among the few missing parts of the almost complete physical map, which is one of the major reasons for cloning and analyzing them. In a first step, by doing *Bal* 31 digestion experiments, we have shown that about 4 to 9 kb of the simple repeated sequence TTAGGC are located at the end of the *C. elegans* chromosomes. This sequence, also present at the *A.lumbricoides* telomeres, shows similarities to the telomeric sequences found in all organisms analyzed so far, including some lower eukaryotes, plants and vertebrates. In order to develop an efficient strategy to clone *C. elegans* telomeres, we have constructed a YAC (yeast artificial chromosome), carrying an *Ascaris* telomere at one end. Upon transformation, we could show that the sequence TTAGGC functions as telomere in yeast, which adds its own telomeric sequences to it. We conclude, therefore, that cloning of *C. elegans* telomeres can be done by complementation of a deficient YAC vector.

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Artificial nucleosome positioning sequences tested in yeast minichromosomes: The strongest rotational signal does not position a nucleosome in vivo.

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Due to the protection of DNA by histone proteins, the position of nucleosomes with respect to the underlaying DNA sequence might play a decisive role in regulation of DNA dependent processes. We have constructed artificial minichromosomes in yeast *S. cerevisiae* to study the sequence dependence of nucleosome positioning. A pentamer of the sequence ($T^{CGG}TG_{TTA}GA^{GCC}TG_{TAA}C$) was reported to be the strongest nucleosome positioning sequence *in vitro* (Shrader and Crothers (1989) PNAS 86,7418). A pentamer and an octamer of this sequence was inserted into the TRP1 gene of a minichromosome in *S. cerevisiae* and the chromatin structures were determined by mapping the accessibility of the DNA to m. nuclease. The artificial sequences did not form precisely positioned nucleosomes *in vivo*, nor did they act as a boundary and shift nucleosome positions on TRP1. Since the TG-sequence contains a rotational signal only, this rotational signal is not sufficient to position the nucleosome *in vivo*.

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CHROMATIN STRUCTURE OF THE XENOPUS rRNA GENES

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Using the psoralen technique we have compared the chromatin structure of the rRNA coding and spacer regions of the two related frog species Xenopus laevis and Xenopus borealis. Isolated nuclei from tissue culture cells were photoreacted with psoralen and the extent of crosslinking in the different DNA regions was analyzed using a gel retardation assay. Both species showed basically the same following results: Restriction fragments from the coding region were resolved into two bands indicating the presence of an active and an inactive population of rRNA genes. A similar crosslinking pattern was obtained with restriction fragments from the spacer promoter/enhancer region. Analysis of fragments including this region and the upstream portion of the gene suggests that each active rRNA gene is preceded by an open spacer promoter/enhancer region.The spacer region downstream of the gene gave a different result indicating the presence of chromatin segments with a heterogeneous psoralen accessibility. This could be an evidence that the RNA polymerases pass the 3' end of the gene and terminate at heterogeneous sites further downstream.

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CHROMATIN ELIMINATION IN *ASCARIS LUMBRICOIDES* TAKES PLACE AT VERY PRECISELY DEFINED CHROMOSOMAL SITES

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During the process of chromatin elimination in *A. lumbricoides*, about 25% of the germ line DNA is expelled from the presomatic cells. The heterochromatic ends of the germ line chromosomes break off and eventually become degraded in the cytoplasm, whereas the shortened chromosomes are distributed to the daugther cells of the elimination mitoses and their descendants. Since we expected the somatic telomeres to be involved in the elimination process, we designed a cloning strategy to isolate such sequences from the somatic genome of *A. lumbricoides*. One of the positive clones obtained, pTel 1, was selected for further analysis. It carries at its end 27 repeats of the *Ascaris* telomeric hexamer TTAGGC, while the subtelomeric DNA sequences are unique and thus specific for a single somatic telomere. The corresponding region within the germ line genome is located at an internal chromosomal site rather than on a telomere. Elimination occurs within this region on a very specific DNA segment; however, it does not take place at a single locus, but rather at many sites. The DNA sequence of this region is very AT-rich, but so far no evidence for the existence of specific elimination signals could be identified. Chromosomal breakage is followed by the addition of tandem arrays of the telomeric repeating units TTAGGC, not present on the germ line chromosome at this site.

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NUCLEOLAR CHROMATIN STRUCTURE AND ITS RELATION TO rRNA SYNTHESIS

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The ratio of active and inactive ribosomal (r) gene copies was determined in regenerating liver cells, in murine lymphosarcoma cell line (P 1798) and in various adult mouse tissues.In rat liver nuclei the number of active r-gene copies remained constant even though their rRNA synthesis increased after partial hepatectomy.In P1798 cells after suppression of rRNA synthesis with a glucocorticoid the number of active r-genes also remained constant. Comparing nuclei of various tissues in adult mice we found differences in the amount of active r-gene copies and these numbers correlated with their run-on activities.We suggest that the cell type i.e. cell differentiation determines the ratio of active and inactive r-gene copies and this in turn determines the maximal possible RNA polymerase I loading of these genes.

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VARIEGATION IN DROSOPHILA : A ZINC-FINGER PROTEIN CONTROLS INACTIVATION OF CHROMOSOME DOMAINS

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Position-effect variegation is the inactivation in some cells of a gene translocated next to the region of the chromosome that is permanently condensed. The product of the gene *Suvar(3)7* enhances this phenomenon by promoting "packaging" of chromatin into heterochromatin. It encodes a protein with five widely spaced Zinc-fingers. Transcripts are maternal, while the protein is detected through development. The protein is nuclear, phosphorylated and binds tightly to chromatin. Similar sequences are found in frogs, mice and men. Analogy with X chromosome inactivation in female mammals and domain-inactivation models for the regulation of homeotic genes lead us to speculate that this or an analogous mechanism of heritable silencing of defined chromosomal regions is an important epigenetic method of gene regulation.

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A PROTECTIVE ROLE FOR THE XENOPUS LA PROTEIN

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Many patients suffering from autoimmune disorders produce antibodies directed against a cellular protein of 48 kd called the La protein. A key property of this protein is its association with the 3' end of all newly synthesized RNA polymerase III (Pol III) transcripts. To test La's possible role in Pol III termination, we have cloned a full-length La cDNA from *Xenopus laevis* into a bacterial expression vector, raised rabbit polyclonal antibodies against the cloned frog protein, and used the antibodies to progressively remove the La protein from S-100 extracts of cultured *X.laevis* kidney cells. Consistent with the results of Gottlieb and Steitz (1989), such immunodepleted extracts generate a reduced number of nascent Pol III transcripts, many of which are shorter than usual, and addition of the bacterial-made La protein partially restores the number of normal length transcripts. But these changes in transcript number and length can be dissociated from Pol III activity because identical results are obtained when RNA generated in a prior reaction with T7 RNA polymerase is incubated in La depleted extracts. Hence, one function of La is to protect nascent Pol III transcripts from nucleolytic degradation.

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SV40 LARGE T-ANTIGEN POSSIBLY MODULATES SPLICING OF CELLULAR PRE-mRNAs

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Onset of SV40 T-antigen synthesis is paralleled by a rapid increase in the steady state levels of 24p3 mRNA (Hraba-Renevey et al. (1989) Oncogene 4, 601-608). This increase could neither be explained by a stimulation of transcription of the 24p3 gene nor by an SV40-induced stabilization of the 24p3 mRNA. The results suggested that the increase in 24p3 mRNA was most likely due to an SV40-induced increase in 24p3 pre-mRNA processing, possibly splicing. Presently we perform *in vitro* splicing reactions with 24p3 pre-mRNA using HeLa cell-free extracts, which efficiently perform splicing of the mouse 24p3 pre-mRNA. To test the hypothesis that SV40 large T-antigen modulates splicing of the 24p3 pre-mRNA, extracts are either prepared from SV40-infected cells, or highly purified SV40 large T-antigen (Dr H. Stahl, Konstanz; Dr. Soussi, Paris) is added to the splicing reactions. Gel retardation assays indicate that SV40 large T-antigen directly binds to the 24p3 pre-mRNA.

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PURIFICATION AND CHARACTERIZATION OF COMPONENTS REQUIRED FOR NUCLEAR PRE- mRNA SPLICING IN VITRO

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Intervening sequences present in nuclear pre-mRNAs are removed from the primary transcript by a process termed splicing. We are investigating this reaction in an in vitro system derived from HeLa cell nuclear extracts. By chromatographic fractionation we obtained five fractions that contain protein factors (SF1, SF2, SF3, SF4, and U2AF) and two fractions which are highly enriched in the major snRNPs U1, U2, U4/U6, and U5.

The function of the different splicing activities in the sequential steps was examined in reconstitution experiments. The formation of the pre-splicing complex (complex A) requires SF1, SF3, U2AF and U1 and U2 snRNPs. This complex is further assembled into an inactive (but functional) splicing complex (complex B) by binding of U4/U5/U6 snRNPs in the presence of SF2. SF4 functions in the conversion of complex B to the active spliceosome (complex C) which is characterized by the presence of the intermediates of the splicing reaction. At least two additional activities are necessary for the production of the final reaction products. One of these factors is present in SF3-containing fractions. Results on the purification and characterization of these factors will be presented.

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POSSIBLE ROLE OF THE 5'POLY(A) LEADER OF LATE VACCINIA VIRUS mRNAS IN TRANSLATION

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All known Vaccinia Virus late mRNAs have a capped poly(A) stretch of 15 to 35 nt as 5'untranslated leader that is not encoded by the viral genome. Therefore it is excluded from hybridization with antisense RNA present late in infection. We are studying the hypothesis that the 5'poly(A) leader may preserve mRNA translatability in supporting translation initiation even in the presence of antisense RNA. To test this hypothesis double stranded RNAs have been synthesized *in vitro* with and without free 5' non coding sequences of varying lengths. Their translation efficiencies have been monitored in wheat-germ extracts and rabbit reticulocyte lysates. Our results show that in these systems a free leader of 81 nt is able to support translation whereas one of 33 nt is no longer preserving translatability. These results might indicate a more complex role of the 5'poly(A) leader in translation. Further analysis testing a possible active role of vaccinia virus are under way.

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ANALYSIS OF THE TRANSLATION INITIATION FACTOR 4A FROM S. CEREVISIAE, S. R. Schmid, P. Buser, C. Elke, A. Prat and P. Linder, Biozentrum, Basel

The eukaryotic translation initiation factor 4A (eIF-4A) is encoded in yeast by two genes, TIF1 and TIF2, which code for exactly the same protein but have highly divergent 5' and 3' flanking sequences. The eIF-4A protein is a member of a family of putative RNA helicases (D-E-A-D proteins). The mammalian factor shows RNA-dependent ATPase activity and functions together with eIF-4B as a RNA helicase.
Using a conditional system we have shown that eIF-4Ay is needed in a in vitro translation system. With the same conditional system we analyzed a number of mutants in the TIF genes in respect to growth rates and in vivo protein synthesis. The analysis of expression shows that in a wild-type cell under standard conditions (full media, 30°C) TIF2 is expressed 5 times more than TIF1 on the translational level. In another approach we tested the function of the two highly homologous mouse 4A proteins in yeast. Expression of the mouse eIF-4AI cDNA cannot complement a temperature-sensitive mutation in yeast eIF-4A.

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Physical interactions between histone pre-mRNA and nuclear factors during 3' end formation

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Different members of the mouse histone gene family vary at least 7.7-fold in their efficiency of RNA 3' processing in vitro. For a pair of differently processed H4 genes, this difference was shown to be due to the so-called spacer element, which, together with the conserved hairpin loop element, forms the genetic signal for RNA processing. The spacer element is known to interact by base-pairing with the RNA moiety of the U7 snRNP, for which we have recently isolated a functional gene and two pseudogenes. For a series of 12 different pre-mRNAs, the efficiency of processing was shown to correlate with the calculated free energy of base-pairing between histone pre-mRNA and U7 snRNA. This computer analysis revealed that, in contrast to previous assumptions, the base-pairing potential extends right up to the processing site, suggesting a biphasic model of U7:pre-mRNA interactions. We have also developed a biochemical assay to analyse the interactions of histone pre-mRNA with the U7 snRNP complex. Preliminary results obtained with this assay have revealed a structural modification of U7 snRNPs in G1-arrested cells which could be related to the known down-regulation of histone RNA 3' end formation in these cells.

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STUDY OF A GUG INITIATION CODON IN THE hPIV1 P/C GENE

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The P genes of Paramyxoviruses produce multiple proteins from alternate reading frames. For Sendai virus, the first initiation codon is a non-AUG (ACG) which starts the C' protein, the second an AUG which starts P, and the third, an AUG which starts C. hPIV1 is very closely related to Sendai, but does not contain the upstream ACG. Nevertheless, a C' protein is made both in vivo and in vitro and appears to be initiated from an upstream GUG codon, located 4 codons upstream from the position of ACG in the Sendai gene and in an optimal context for ribosomal initiation (ACAGUGG). To confirm the upstream GUG as the initiation codon for the hPIV1 C' protein, three mutants were created and their translation was studied in vitro. When the GUG was changed to AUG, C' showed a much higher level of expresion relative to the P and the C proteins, whereas no C' synthesis could be detected when the GUG was replaced by GCG. Finally, the level of expression of C' was dramatically reduced when the favorable context for initiation was modified by replacing the A at position -3 (the first nucleotide of the initiation codon being +1) with a T. This is the first natural example of a GUG initiation codon.

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ANALYSIS OF THE MECHANISM OF PARAMYXOVIRAL EDITING

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Transcription of paramyxoviral phosphoprotein (P) genes yields normal and edited mRNAs. The latter contain G residues at a defined position, probably added by polymerase stuttering. In measles (MV) and Sendai virus, normal transcripts encode P, edited transcripts containing preferentially one added G, encode V. In mumps and simian virus 5 (SV5), normal mRNAs encode V, edited transcripts containing preferentially two added Gs, encode P. A model to explain preferential insertion of either one or two Gs, respectively, was proposed by Vidal et al. (EMBO J. 9 (1990) 2017-2022). Changing the sequence of the MV P gene around the editing site to that of mumps or SV5, according to this model, should result in the preferential insertion of two Gs in the MV P mRNA. Thus, six different variants of the editing site were introduced in an "infectious" MV cDNA clone (EMBO J. 9 (1990) 379-384), and rescue of mutants which edit in the mode of mumps or of MV will be attempted. If infectious viruses can be rescued, analysis of the mRNAs will define the mode of stuttering.

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THE P GENE OF BOVINE PARAINFLUENZA VIRUS 3 EXPRESSES ALL THREE READING FRAMES FROM A SINGLE mRNA EDITING SITE

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The P gene of bPIV3 contains two downstream overlapping ORFs, called V and D. By comparison with the mRNA editing sites of other paramyxoviruses, two editing sites were predicted for bPIV3; site a to express the D protein, and site b to express the V protein. Examination of the bPIV3 mRNAs, however, indicates that site b is non-functional whereas site a operates frequently. Insertions at site a give rise to both V and D protein mRNAs, because a very broad distribution of Gs are added when insertions occur. This broad distibution is very different from the editing sites of Sendai virus or SV5, where predominantly one form of edited mRNA containing either a 1 or 2G insertion respectively is created, to access the single overlapping ORF of these viruses. A model is proposed to explain how paramyxoviruses control the range of G insertions on that fraction of the mRNAs where insertions occur. The bPIV3 P gene is unique as far as we know, in that a sizeable portion of the gene expresses all 3 reading frames as protein. bPIV3 apparently does this from a single editing site by removing the constraints which control the number of slippage rounds which take place.

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SELF-SPLICING AND MOBILITY OF A GROUP I INTRON IN THE CHLAMYDOMONAS REIHARDTII CHLOROPLAST

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The chloroplastic 23S ribosomal RNA gene of the green unicellular alga *Chlamydomonas reinhardtii* harbours a group I intron which contains an internal open reading frame (ORF). A precursor encompassing the intron with its 5' and 3' flanking sequences was shown to undergo self-splicing during *in vitro* transcription resulting in an excised intron and joined exons. Isolated precursor, corresponding to the full-length *in vitro* transcript, is capable of self-splicing *in vitro* also in the absence of protein factors.
When the internal ORF was expressed in *E.coli* in the presence of a second plasmid which contained a cDNA corresponding to the intronless form of the 23S rRNA gene, specific cleavage close to the exon-exon-junction was observed.
Chloroplasts of *C.reinhardtii* were transformed with the same cDNA as a model for an intronless copy of the 23S rRNA gene. All the transformants which had the cDNA integrated at the expected site in the chloroplast genome had also the intron inserted into this cDNA.
These experiments show clearly that the intron of the chloroplastic 23S rRNA gene of *C.reinhardtii* behaves as a ribozyme at the RNA level and as a mobile genetic element at the DNA level.

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A SMALL CHLOROPLAST RNA IS REQUIRED FOR TRANS-SPLICING IN CHLAMYDOMONAS REINHARDTII.

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The three exons of the *psaA* mRNA are transcribed separately from widely scattered loci on the chloroplast DNA of *Chlamydomonas reinhardtii*. The three separate transcripts are then assembled by a process involving *trans*-splicing. At least one additional chloroplast locus and fourteen nuclear genes are required for psaA maturation. The chloroplast gene (*tscA*) involved in *trans*-splicing of exons 1 and 2 has been mapped by particle gun transformation of a deletion mutant. The 0.7kb region of the chloroplast genome which is sufficient to rescue *tscA* function has been subjected to insertion mutagenesis, showing that it does not contain significant open reading frames. These experiments indicate that the product of the *tscA* gene is a small chloroplast RNA, which acts *in trans* in the first *trans*-splicing reaction of *psaA*. In a model for the mode of action of this RNA, the characteristic structure of group II introns is assembled from three separate transcripts.

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RECONSTITUTION OF AAUAAA-DEPENDENT MESSENGER RNA POLYADENYLATION FROM PURIFIED COMPONENTS IN VITRO

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Polyadenylation of mRNA depends on the sequence AAUAAA located close to the 3' end. We have reconstituted this reaction from purified components in vitro. Poly(A) polymerase was purified 6000fold to homogeneity from calf thymus. The enzyme (MW 60 kD) is nonspecific with respect to the primer. Activity is much higher in the presence of Mn^{++} as opposed to Mg^{++} due to a 100fold higher affinity for the primer terminus. In the presence of Mg^{++}, a second factor (cleavage and polyadenylation factor, CPF) specifically permits polyadenylation of AAUAAA-containing RNAs. CPF was purified to near homogeneity from calf thymus as a complex of four proteins of 160, 100, 70, and 30 kD. It interacts directly with the AAUAAA sequence. Although poly(A) polymerase and CPF are sufficient, AAUAAA-dependent polyadenylation is strongly stimulated by poly(A) binding protein. In the presence of this protein, a biphasic polyadenylation is observed: First an oligo(A) tail is added which is then rapidly elongated. Poly(A) binding protein allows CPF- and AAUAAA-independent elongation of an oligoadenylated RNA.

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PURIFICATION AND CHARACTERIZATION OF YEAST POLY(A) POLYMERASE

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In yeast, the factors and sequences involved in mRNA 3' end formation are different from those in higher eucaryotes and the mechanism of 3' processing is not known.

We have purified poly(A) polymerase from a whole cell extract of *Saccharomyces cerevisiae* to homogeneity. The enzyme is a monomeric polypeptide with a denatured molecular weight of 63,000. The activity of the enzyme is higher with manganese than with magnesium as divalent cations. Various RNA homopolymers as well as tRNA or rRNA can serve as primers. An RNA that terminates at the natural poly(A) site of the *CYC1* gene is not more efficiently elongated than several nonspecific substrates, indicating the requirement for additional factors to provide specificity. Elongation of the primer is distributive. Covering of poly(A) primer with poly(A) binding protein reduces the enzyme's activity more than tenfold.

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CARACTERIZATION OF THE IRON REGULATORY FACTOR (IRF), A CYTOPLASMIC mRNA- BINDING PROTEIN

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IRF binds specifically to conserved iron responsive elements (IREs) both in ferritin and transferrin receptor (TR) mRNAs. Binding of this regulatory factor leads to inhibition of ferritin translation and an increase in TR mRNA stability. We have affinity purified IRF from human placenta by taking advantage of its specific RNA binding properties. The purified factor migrates as a 95/100 kD doublet on SDS-PAGE. Partial V8 protease digestion of the natif, affinity purified protein generates a 63/68 kD doublet and a 31 kD fragment. The N-terminal sequence obtained from the 31 kD fragment is identical with a recently published cDNA clone of IRF. UV crosslinking experiments using partial V8 protease-digested and trypsin-digested IRF indicate that RNA binding does not require a C-terminal portion of the protein.

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S. CEREVISIAE TRANSLATION INITIATION FACTOR 4A

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In eukaryotic cells the binding of ribosomes to mRNA is mediated by initiation factors of the eIF-4 group. They guide the ribosome to the 5' region of the mRNA, melt mRNA secondary structure and facilitate scanning of the mRNA by the ribosome and selection of the correct AUG for translation initiation. A member of the initiation factor 4 family, eIF-4A, is a RNA helicase which is able, together with eIF-4B, to melt double-stranded RNA in an ATP-hydrolysis-dependent manner. We have isolated eIF-4A from the yeast *Saccharomyces cerevisiae* and developped a cell-free translation system which is dependent on exogenous eIF-4A for translation initiation. This system is suitable to study the eIF-4A requirement for translation of individual mRNAs. Furthermore, it can be used to study structure-function relationships of eIF-4A by expressing different mutant yeast eIF-4A in *E. coli* and testing these factors in the eIF-4A-dependent cell-free translation system.

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SPECIFIC MARKER EFFECTS AND MISMATCH REPAIR IN *S.POMBE*

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Intragenic two-factor crosses between the G->C transversion allele *ade6-M387* and nearby *ade6*-mutations give rise to highly increased frequencies of prototrophic recombinants compared to the same crosses with the C->T transition allele *ade6-51* located only four base pairs upstream of M387.
A detailed genetic analysis of this marker effect showed that the increase of the prototrophic frequency is dependent on the map position of the second allele in the cross. The effect is strong (->30 times) for alleles close to M387/51 (+/-30bp) and quickly decreases with increasing map distance (absent >200bp).
As for the G->C transversions in *sup*-tRNA genes of *S.pombe* an increased PMS frequency of 10 fold compared to *ade6-51* has been confirmed also for M387, establishing a connection with mismatch repair. The measured PMS/conversion ratios at different loci determine a C/C mismatch repair efficiency of about 70% in *S.pombe*.
We suggest the presence of an inefficient very short patch repair system processing the C/C mispairs and thus allowing independent repair over short distances, to account for the marker effect and further the existance of two additional mismatch repair pathways producing different excision tract length, to explain the characteristic distance dependence of the marker effect.

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A SPECIFIC DNA SEQUENCE IS REQUIRED FOR HIGH FREQUENCY RECOMBINATION IN THE *ade6* GENE OF *S. POMBE*.

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The M26 mutation is a G to T base substitution 136 nucleotides from the 5' end of the *ade6* gene. It enhances recombination frequencies by an order of magnitude in comparison with other mutations.(e.g. the mutation M375 three base pairs upstream of M26). Increased recombination is observed in intragenic two-factor crosses and in tetrad anlysis of one-factor crosses with M26 (5% conversion tetrads versus 0.25 to 0.9 % with other *ade6* mutants). - The hypothesis was tested that this hotspot of recombination requires a specific nucleotide sequence at the M26 site. The DNA sequence was altered systematically by *in vitro* mutagenesis, and the resulting sequences were introduced into the *ade6* gene *in vivo* by gene replacement. It results that any change of the heptanucleotide ATGACGT (including the M26 site) leads to loss of high frequency of recombination. Thus this sequence is necessary for hotspot activity, but it seems not to be sufficient. Preliminary data indicate that a protein may bind to this sequence.

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STIMULATION OF RECOMBINATION BY A STRONG TRANSCRIPTION PROMOTER AND ANALYSIS OF CONVERSION TRACTS IN THE *ade6* GENE of *S.*POMBE

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The weak promoter of the *ade6* gene was replaced by the strong promoter of the *adh1* gene *in vivo*. Increased mRNA levels were observed in mitotic and meiotic cells. Intragenic recombination in *ade6* was enhanced 7-fold in mitosis and 20-25-fold in meiosis (conversion and crossover). Enhancement of recombination by the *adh1* promoter is partially additive with enhancement by the hotspot mutation M26.

Five restriction site polymorphisms were introduced into the 5' region and the *ade6* gene. From several two-point crosses (with or without the hotspot allele M26) wild-type recombinants were isolated and the convertants not associated with crossovers selected with the help of flanking markers. The length of individual conversion tracts was determined. The pattern of conversion tracts is not affected by M26. It is concluded that M26 enhances initiation (not termination) of recombination at a preexisting site.

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REGULATORY MUTANTS AFFECTING THIAMINE METABOLISM IN *SCHIZOSACCHAROMYCES POMPE*

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The role of thiamine (vitamine B1) in gene regulation is investigated. In this context mutants of *S.pombe* defective in the regulation of thiamine repressible acid phosphatase have been genetically mapped and characterized in terms of thiamine metabolism. The results suggest the following:
1. Genes tnr1, tnr2 and tnr3 act negatively and gene thi1 positively on the expression of the genes pho4 (coding for thiamine repressible acid phosphatase) and thi3 (coding for a thiamine biosynthetic enzyme and called nmt by Maundrell).
2. Tnr1, tnr2 and tnr3 affect thiamine transport negatively, thi1-1 positively.
3. Genes thi1 and tnr3 are regulating the size of the intracellular thiamine pool.

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PROTEINS FROM YEASTS INVOLVED IN RECOMBINATION

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Hybrid DNA is the central intermediate in homologous recombination. Using biochemical assays, activities that can form hybrid DNA *in vitro* have been identified in a wide variety of organisms including fungi, flies, man, and plants. The strand exchange protein 1 (SEP1) from *S.cerevisiae* has been previously purified to homogeneity as a M_r 132,000 polypeptide sharing many biochemical properties with the *E.coli* recA protein, the central recombination protein in this organism. Thus a role for SEP1 in yeast recombination is inferred. The native form of SEP1 is a M_r 175,000 polypeptide containing strand exchange activity as shown by immunological, biochemical, and molecular studies . The gene encoding SEP1 has been cloned, but the primary sequence does not reveal any significant sequence homologies to other proteins in data base searches. SEP1 is an abundant cellular protein with presumably nuclear localization and is present throughout the mitotic cell cycle and meiosis.

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DIRECTED CHLOROPLAST TRANSFORMATION IN *CHLAMYDOMONAS REINHARDTII*: INSERTIONAL INACTIVATION OF THE *PSAC* GENE.

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The 8-9 kDa bound iron sulfur protein plays a role as terminal electron acceptor of the photosystem I (PS I) and is encoded by the chloroplast gene *psa*C. To examine the role of this protein, *psa*C was inactivated by chloroplast transformation. Using a particle gun, wild type *C. reinhardtii* cells were transformed with a plasmid carrying *psa*C disrupted by an aadA cassette gene designed to express spectinomycin and streptomycin resistance in the chloroplast. Transformants selected on plates containing acetate and spectinomycin were unable to grow on plates of minimal medium lacking acetate and were shown to specifically lack PS I activity. Southern blot analysis of total cell DNA showed that the wild type *psa*C in the chloroplast genome had been replaced by the disrupted *psa*C through homologous recombination. Biochemical analyses indicated that no subunits belonging to the PS I reaction center accumulate stably in the thylakoid membranes of the transformants. We conclude that the bound iron sulfur protein is an essential component both for photochemical activity and stable assembly of the PS I complex. The present study suggests that any chloroplast gene encoding a component of the photosynthetic apparatus in *C. reinhardtii* can be disrupted using the strategy described.

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ANTISENSE RNAS AGAINST THE SMALL SUBUNITS OF PHOTOSYSTEM I IN *CHLAMYDOMONAS REINHARDTII*

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To study the role of the small subunits of the photosystem I complex (one of the three complexes of the thylakoid membranes, involved in electron transfer from water to $NADP^+$), in the green alga *Chlamydomonas reinhardtii*, we have inhibited their expression by antisense RNAs. Four cDNA sequences were fused in their antisense orientation to the promoter of the gene of the small subunit of ribulose bisphosphate carboxylase and introduced into *arg7cw15 C. reinhardtii* cells by transformation using the argininosuccinate lyase gene as selective marker. Northern hybridizations revealed that the level of the corresponding sense transcripts of the photosystem I subunit genes are reduced in the transformants. Amongst the antisense transformants, we found one clone that displays the characteristics of a photosystem I mutant. Analysis of this putative nuclear mutant may allow us to isolate a new gene involved in photosystem I activity.

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Analysis of the *Staphylococcus epidermidis mec*R locus, which regulates the expression of *mec*A, the gene essential for methicillin resistance in staphylococci

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The *mec*A gene coding for the penicillin binding protein 2' (PBP2') in methicillin resistant staphylococci, resides on the methicillin resistance determinant (*mec*), which is not present in sensitive strains. This PBP2' is essential for the expression of methicillin resistance (Mc^r) in staphylococci. The cloned *mec*A gene of both organisms can confer methicillin resistance to Mc^S staphylococci. (Tesch et al., 1990). Further it has been shown that PBP2' was inducible by methicillin (Ubukata et al.,1985; Tesch et al., 1989; Ryffel unpublished results). Tesch (1989) succeeded in cloning the *mec*R locus of *S. epidermidis*, a DNA region linked to the *mec* determinant, which coded for factor(s) able to down-regulate the expression of *mec*A and the Mc^r phenotype. By *Bal*31 deletion analysis we specified a 2.2 kb DNA fragment, essential for the repression of *mec*A. Sequence and Northern blot analysis of the *mec*R locus, revealed that this DNA region codes for at least two open reading frames, ORF520 and ORF110, which shared strong amino-acid similarities to *bla*R1 and *bla*I genes, the regulatory elements of the *bla* regulon in *Bacillus licheniformis*.

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GROWTH IN THE PRESENCE OF METHICILLIN OR THE INACTIVATION OF *FEMA* HAVE SIMILAR EFFECTS ON AUTOLYTIC ACTIVITIES IN HOMOGENEOUS METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*.

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Upon growth in the presence of methicillin, isolated crude cell wall- and extractable-autolytic activity increased in Col and DU4916. In their respective *fem*A$^-$ mutants BB403 and BB401 these activities were also increased. Methicillin had no effect on whole cell lysis in Col and DU4916. However, whole cell lysis was slower in BB403 compared to Col and faster in BB401, compared to DU4916. Though whole cell lysis in Col and DU4916 differed, their crude cell wall- and extractable- autolytic activities were similarily affected by growth in the presence of methicillin and upon inactivation of *fem*A, probably resulting from similar changes in cell wall structure. Autolytic banding patterns of freeze-thaw extracted autolysins, revealed no major difference in Col and BB403 and in DU4916 and BB401.

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FUNCTION OF ATP HYDROLYSIS DURING THE RECA-MEDIATED RECOMBINATION REACTION

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We recently demonstrated that RecA can promote base-pair switching between completely homologous double- and single-stranded DNA molecules even in the absence of ATP hydrolysis (J.Mol.Biol.**215**, 1990, in press). Our present studies indicate that RecA needs the energy of ATP hydrolysis to promote the reciprocal strand exchange between two double-stranded regions of the interacting DNA molecules. The RecA-promoted strand exchange between double- and single-stranded DNA also seems to need the energy of ATP hydrolysis to proceed over the regions of heterology between partially homologous DNA molecules. These findings shed light on the different mechanisms by which RecA promotes reciprocal and non-reciprocal strand exchange reactions and how it can promote branch migration over heterologous regions of the interacting DNA molecules.

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DOWNY MILDEW OF *Arabidopsis*: ISOLATION OF HOST GENES INVOLVED IN RESISTANCE

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Recently, *Peronospora parasitica* infection of a local (Weiningen near Zuerich) strain of *Arabidopsis thaliana* was observed, and subsequently the Landsberg erecta ecotype was also shown to be susceptible. By 18 hours after inoculation, well developed intercellular hyphae with intracellular haustoria were visible. In contrast, the RLD and Columbia strains of *Arabidopsis* are highly resistant to the same isolate of *P. parasitica* and respond with a typical hypersensitive reaction (HR). Crossing experiments between resistant and susceptible ecotypes of *Arabidopsis* suggest that the resistance trait is inherited in a Mendelian fashion and experiments to map the resistance locus are in progress. The importance of *Arabidopsis* is that its relatively small genome (approximately 100 000 kbp) and low amount of repetitive DNA make it feasible to use a map-based cloning strategy to isolate the resistance locus. Similarly, the investigation of mutants altered in their response to the pathogen should enable the cloning of genes from the putative signal transduction pathway, involved in the activation of defence genes after pathogen perception, and genes involved in defence responses *per se*.

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FACTORS DETERMINING THE COINTEGRATION FREQUENCY OF INSERTION SEQUENCE IS*3* OF *E.COLI*.

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Cointegration activity of the insertion sequence IS*3* of *E.coli*, normally at only about 1 % of the activity of IS*1*, could be strongly enhanced by using plasmid constructions in which a second IS*3* element, mutilated by a large deletion, was placed adjacent to an intact IS*3* copy. The frequency of cointegration was also increased in an *E.coli* strain deficient in Dam methylation. Insertion sites were strongly clustered within the target lambda repressor gene and carried the IS element always in the same orientation; target sequence duplications were mostly 3 bp, but in some cases 4 bp long. To identify the roles of the open reading frames (ORFs) in IS*3*, we inactivated potentially functional ORFs by site-directed mutagenesis. Mutations inactivating the putative initiation codons of ORFs I and II reduced the activity to less than 4 % of the wt sequence, but the introduction of a termination codon into ORF IV had no effect on cointegration frequency. We conclude that translation of ORFs I and II is essential for cointegration activity and that the mutationally changed ATG codons most probably serve as the normal initiation codons in the wt element. In contrast, ORF IV could either be nonfunctional or its gene product could be supplied in trans from chromosomal elements.

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GLIOBLASTOMA SECRETE IL-8, A LEUCOCYTE CHEMOTACTIC FACTOR, IN VIVO AND IN VITRO.

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Glioblastoma patients have a deficient cell-mediated immunity shown by cutaneous anergy, reduced numbers of T cells and decreased lymphocyte reactivity to mitogens. However multiple lymphoïd infiltrates are present on the tumor sections, consisting predominantly of CTL. To get a better understanding of the chemical signals mediating these infiltrations we tested for the presence of chemotactic cytokines. An interesting candidate was IL-8, involved in neutrophil, basophil and T lymphocyte attractions. We found various levels of IL-8 secretion in 6/7 glioblastoma cell lines as analyzed by ELISA or glucosaminidase assays. Specific IL-8 mRNAs were constitutively detected in 14/15 cell lines. Both the mRNA and soluble IL-8 could be increased by IL-1b and TNFa. To assess in vivo IL-8 production we showed that ex vivo extracted tumors contained IL-8 mRNA, both in low and high grade astrocytomas. In complement CSF and cyst fluids of glioblastoma patients were also analyzed. Using immunocytochemistry on frozen sections we could demonstrate that the tumor cells themselves produce IL-8.
Although this study demonstrates in vivo IL-8 secretion, only weak neutrophil infiltrates are found in these tumors. Therefore we analyzed the possible role of inhibitory factors antagonizing this cellular immune response. Influences of IL-8 on the cytotoxicity and adhesion of leucocytes to the tumor cells will also be presented. Finally the secretion appears to be paracrine as no IL-8 receptors were found on 15 glioblastoma cell lines analyzed.

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αB CRYSTALLIN IS A HEAT SHOCK PROTEIN

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We have previously isolated and characterized a protein whose synthesis is transiently stimulated by the Ha-ras and v-mos oncogene products. The protein is identical with αB crystallin isolated from eye lenses. Sequence similarity between small heat shock proteins (hsps) and αB crystallin have prompted us to investigate whether αB crystallin is stress-inducible. We tested three conditions which are well known to induce hsp genes: hyperthermia and the incubation with Cd^{2+} or sodium arsenite. All three stress situations led to the accumulation of substantial amounts of αB crystallin in NIH 3T3 cells. Small hsps aggregate to form spherical 17S particles. These are localized in the cytoplasm and redistribute to a position inside or closely associated with the nucleus at elevated temperatures. We could demonstrate that αB crystallin shares these same physical properties with the small hsps. A DNA fragment flanking the αB crystallin gene at the 5'end was fused to the CAT gene and this construct was introduced into NIH 3T3 cells. A 12 fold increase of the basal CAT activity was observed upon heat treatment. Thus heat-induced expression of the αB crystallin gene is regulated at the transcriptional level.

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Genes and pseudogenes for human heat shock protein HSP65

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Heat-shock protein HSP65 is highly conserved across species and is recognized by specific T cell clones involved in autoimmune pathogenesis. We wanted to analysed HSP65 genes and their potential implication in the developpement of autoimmunity.
Southern blots indicated the presence of several HSP65 genes. Sequence analysis of PCR amplified genomic DNA revealed a familly of related genes, all pseudogenes. In contrast, the published HSP65 sequence could only be amplified from mRNA.
The absence of the normal HSP65 sequence in PCR subclones generated from genomic DNA could be due to either the presence of introns in the expressed HSP65 gene or the large number of related genes. The possibility of allelic diversity within the relevant immunodominant segment of human HSP65 by PCR analysis on total RNA will be explored.

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CIS- AND TRANS-ACTIVE ELEMENTS REGULATING THE ADULT RABBIT ß-GLOBIN GENE.

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The *Xenopus* oocyte system has been used for functional tests of cis- and trans-acting elements involved in tissue-specific regulation of the ß-globin promoter. The trans-active factor has been enriched from a chromatin wash of bone marrow cells by following its activity through successive Sephadex, heparin-agarose, and oligonucleotide-Sepharose affinity columns. The responsive DNA element has been mapped by testing the transcriptional activation of 5'-deleted ß-globin and of mosaic promoters. An imperfect repeat, AAGGCAGAGC-AGGGCAGCTGC, is located between the TATA box and the cap site. This element mediates tissue-specific trans-activation when inserted upstream of a truncated, inactive thymidine kinase promoter (-79) or coding sequence (+55). Transcription initiation takes place just downstream from the inserted element.

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A NEW CLASS OF MTDNA-LIKE FRAGMENTS ASSOCIATED WITH AGING

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A new class of mtDNA-like fragments has been isolated from human liver tissue and fibroblast cell cultures. Various oligonucleotides, specific for the region of human mtDNA coding for the genes of the cytochrome oxidase subunit 3 and NADH dehydrogenase subunit 6, were used in the polymerase chain reaction assay with fragmented nuclear DNA as templates. The results show the existence of numerous mtDNA-like fragments varying in length and restriction enzyme pattern. The difference in length indicates that the original mtDNA has been inserted and possibly rearranged. The appearance of some of the fragments is related to different stages of aging both in cell culture and in liver tissue. Further studies should elucidate the role of these fragments in the cellular aging.

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PCR-ASSISTED CLONING OF A SHIKIMATE KINASE cDNA FROM TOMATO

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Shikimate kinase (EC 2.7.1.71) catalyzes the fifth step in the biosynthetic pathway of the aromatic amino acids. This shikimate pathway occurs exclusively in plants, fungi and microorganisms. We report on the first isolation of a shikimate kinase specific cDNA of a higher plant.
Two degenerated oligonucleotides, corresponding to highly conserved sequences of known microbial shikimate kinases have been used for a Polymerase Chain Reaction (PCR) with ss cDNA as template. Fragments of the expected size were subcloned and sequenced. The deduced amino acid sequence of one fragment showed a high homology to the known shikimate kinase sequences and was used as a probe to screen a tomato cDNA library. Eight positive clones have been isolated. Results of the analysis will be presented.

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Identifying the gene sequences for RNases using the polymerase chain reaction

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We have identified conserved sequence elements in the genes for diverse members of the ribonuclease families and used these to design paired oligonucleotide primers for the polymerase chain reaction (PCR). Using such probes, it was possible to determine most of the coding sequence for the hamster pancreatic gene directly from genomic DNA in one experiment. The same strategy can be used for determining the gene sequence of the pancreatic genes from other organisms. These sequences may help establish the evolution of the pancreatic RNase gene family. However, our major interest is to identify the genes for RNases that may be involved in the intracellular control of mRNA levels, as much less is known about these proteins than their pancreatic counterparts. We are therefore also screening the hamster DNA using probes based on the gene sequences for human angiogenin, eosinophil neurotoxin, and eosinophil cationic protein. These proteins, although functional RNases, were all identified by their ability to affect the growth of mammalian cells. Probes based on the two genes from human eosinophils have detected at least one related sequence in hamster DNA. The PCR products can be directly cloned or labeled and used as probes to screen libraries.

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INTRONIC SEQUENCES CONTROL IMMUNOGLOBULIN HEAVY CHAIN EXPRESSION IN TRANSGENIC MICE

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Evidence is emerging that sequences other than the well characterized intronic enhancers contribute to high expression levels of immunoglobulin genes in vivo. We observed that the heavy chain of a chimeric antibody of the human IgG1 isotype is poorly expressed in the serum of transgenic mice, although its transcription is controlled by the immunoglobulin-promoter and -enhancer. Addition of sequences from the major intron of the heavy chain to the gene construct raised the expression of the human IgG1 heavy chain by a factor of 1000 to approximately 100-1000 µg/ml in the serum of transgenic mice. High expression of the transgene is reflected by increased amounts of human IgG1 mRNA in the spleen and lymphnodes.

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GENE THERAPY FOR DROSOPHILA ACETYLCHOLINESTERASE

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Acetylcholinesterase (AchE) interrupts cholinergic neurotransmission by hydrolyzing acetylcholine in the synaptic cleft. In Drosophila, AchE is found primarily in the central nervous system as an amphiphilic dimer linked to the membrane by a glycolipid anchor. The locus (Ace) has been cloned and sequenced in our laboratory. Its structure reveals a 34 transcription unit split in 10 exons . The 1086 base long leader contains six small open reading frames which could negatively regulate translation (as for yeast GCN4).
The 34 kb transcription unit is too long for efficient P-mediated genetic transformation. Using a combination of cDNAs and 1.5 Kb of genomic upstream sequences, we constructed a 5.9 kb minigene. This intronless construct rescues Ace lethal mutations, as does another shorter construction with a deletion of the small ORFs. The distribution of AChE activity in flies with the two differents constructs cannot be distinguished from wild-type.

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AUTOREGULATION OF THE *FTZ* GENE: FUNCTION AND MECHANISM

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The *Drosophila melanogaster* segmentation gene *fushi tarazu (ftz)* is transcribed in seven stripes in the developing embryo. The *ftz* upstream element, a 2.6 kb enhancer located 3.5 kb upstream of the *ftz* transcription start site, is required for correct *ftz* expression. The upstream element drives reporter gene expression in seven *ftz* -like stripes in trangenic flies. Interestingly, this expression is fully dependent on the presence of the *ftz* gene product itself. This suggests that the upstream element mediates an autoregulatory feedback loop that maintains the expression of *ftz*.
The upstream element contains two independently autoregulated enhancer elements that both direct striped expression in a germ-layer restricted manner. *In vitro*, several regions within these elements are bound by purified *ftz* homeodomain. Mutations of these *ftz in vitro* binding sites lead to a strong reduction of reporter gene expression *in vivo*. Furthermore, several of these binding sites are also conserved in the *ftz* homolog of *D. virilis*, a *Drosophila* species that diverged about 60 million years ago from *D.melanogaster*. The genetic, biochemical, evolutionary, and *in vivo* expression data suggest that *ftz* directly regulates its expression by binding in combination with other transcription factors to the *ftz* upstream element.

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MOLECULAR AND GENETIC ANALYSIS OF *SLOPPY PAIRED*, A PAIR RULE GENE IN *DROSOPHILA MELANOGASTER*

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The *sloppy paired (slp)* gene is involved in the establishment of the metameric body plan of the *Drosophila* embryo. We have cloned the *slp* locus by means of a recently developed method, i.e. P-element-mediated enhancer detection. We recovered two enhancer detector transposon insertions in the *slp* locus that enabled us to recover four additional *slp* alleles by mobilizing the transposon and screening for imprecise excisions. A detailed phenotypic and molecular study of the new alleles will be presented. We detected two transcription units in the *slp* locus both of which may contribute to the *slp* phenotype. Sequencing of cDNA and genomic recombinant phages from the *slp* locus revealed that the predicted amino acid sequences of the two proteins share considerable homology. A domain consisting of 77 amino acids is also found in the *Drosophila forkhead* protein and HNF-3A, a liver enriched transcription factor. It has been proposed that this novel motif, the ***forkhead* domain**, is involved in DNA binding.

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A NOVEL APPROACH FOR IDENTIFYING *ANTP*-REGULATED GENES

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Homeotic genes specify the unique identities of the different body segments of Drosophila melanogaster. These homeotic genes act as selector genes that are thought to initiate specific developmental pathways by controlling the expression of genes that are involved in structural and cellular differentiation.
Localized expression of the homeotic gene Antennapedia (Antp) is required to initiate normal development in the thoracic segments of the embryos and adult flies. When the Antp gene is expressed ectopically in the larval primordium of the antenna (the antennal imaginal disc) the developmental fate of the disc is switched and the antenna of the adult is transformed to a mesothoracic leg. We have screened about 500 fly strains carrying single copies of an enhancer detector transposon, to identify regulatory elements or genes whose activity in antennal discs is modified in response to this transformation. Several regulatory elements that are either directly or indirectly regulated by the Antp protein were found. The expression in the antennal disc of one particular enhancer detector strain is strongly repressed by Antp. The transposon is located near a similarly regulated gene in the spalt locus, a locus which encodes a homeotic function in embryonic head and tail development.

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Empty spiracles, a Homeobox Gene of *Drosophila melanogaster* Involved in Head Development

Walldorf, U., Kloter, U. & Gehring, W.J.

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THE EXPRESSION OF THE MYELOPEROXIDASE (MPO) GENE IS SPECIFICALLY DOWN-REGULATED DURING HL60 DIFFERENTIATION

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In the presence of phorbolmyristate acetate (PMA) the leukemic HL60 cell line is induced to differentiate to macrophage-like cells. In parallel a down-regulation of the MPO transcript to an undetectable level is observed within 24 hrs. Simultanously the CD18 mRNA, coding for the common subunit of an integrin subfamily increased as demonstrated by Northern blot analysis. These two regulatory events took place during a time period when overall protein synthesis did not change significantly. Furthermore, the complexity of the gene expression pattern for induced/uninduced cells is quite similar. Therefore, these data suggest a specific down-regulation of MPO expression. The effect might be mediated by fos and jun. PMA acted as an inducer even if it was present for only 10 min before being washed out. As cell differentiation is accompanied by a general cell death, the initiation of apoptosis (programmed cell death) cannot be excluded at this time.

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The minute virus of mice capsid specifically recognizes the 3'-hairpin structure of the viral replicative form DNA:mapping of the binding site by hydroxyl radical footprinting

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The terminal hairpin structures of the minute virus of mice (MVM) DNA are essential for viral replication. Here we show that the hairpin 3'-terminus of MVM RF DNA interacts specifically with empty MVM capsids. No binding for the same terminal sequence in its extended conformation was found. Hydroxyl radical footprinting analyses reveal three capsid binding sites around the branch point of the two 3'-terminal hairpin arms looping out from the DNA stem (T-structure). Single base changes within these sites do not effect the binding. Band-shift experiments with purified viral proteins demonstrate a specific binding affinity for VP1 but not for VP2.

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CHROMATIN REPLICATION *IN VITRO*

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TEMPLATE FUNCTION OF MODIFIED Qß RNA's FOR BACTERIOPHAGE Qß REPLICASE

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In vitro transcribed full-length bacteriophage Qß RNA of both polarities altered only at the 3' termini as well as variants with normal ends but an internal deletion or insertions (kanamycin- or tetracyclin-resistance genes) were used as templates for Qß replicase. Surprisingly, RNA's with shortened or overlength 3' ends were active templates, although at low efficiency. Initiation took place at or close to the physical 3' end and not internally at the natural Qß 3' terminal region. Among the series of templates containing an internal deletion or insertions, the minus strand variants exhibited reduced initiation efficiencies whereas, unexpectedly, the plus strand variants showed even enhanced initiation. However, elongation slows down dramatically in the region of the foreign insert. This slow elongation is accompanied by an increased tendency of the product strand to hybridize to the template as revealed by a 2.5 fold increase of RNase resistance as replicase passes from Qß-specific to insert sequences.

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A NOVEL MECHANISM FOR THE INITIATION OF TACARIBE ARENAVIRUS GENOME REPLICATION.

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Tacaribe virus (TAC) is a member of the family Arenaviridae, a di-segmented negative strand RNA virus. The genomes replicate via full-length complementary strand intermediates which we refer to as antigenomes. For all other negative strand viruses, the 3' end is a U and replication initiates at the precise 3' end with ATP. For Arenaviridae, the 3' end is a G, meaning that replication should initiate with CTP, which would be unique. We have studied the 5' ends of TAC genomes and antigenomes and have found that they were not capped and that replication initiates on an extra G in position -1. This extra G is not templated by a C on the 3' end of the complementary molecule. The study of the 5' end of TAC mRNAs transcribed from both genomes and antigenomes have shown that these messengers are capped and that transcription initiates with 1 to 5 non templated extra nucleotides with heterogeneous sequences but never a single G at position -1 as for the replication. We have developed an in vitro system and shown that among diferent di- or tri-nucleotides tested, only 2, GpC and ApApC, appear to stimulate both viral transcription, replication and encapsidation. A model is proposed in which genome replication initiates with pppGpC to create the non templated extra G.

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ROLE OF HUMAN CTF/NF-I IN ADENOVIRUS TYPE 2 DNA REPLICATION IN VITRO.

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In order to study the role of CTF/NF-I in the activation of Adenovirus-2 (Ad) DNA replication, we have developed an in vitro assay that allows reconstitution of the initiation of replication using, in addition to the Ad genome, three proteins expressed in rabbit reticulocyte lysate, 1) Ad DNA polymerase (AdPol), 2) Ad preterminal protein (pTP), the covalent acceptor for protein-primed initiation and, 3) CTF-1, a member of the CTF/NF-I human DNA binding proteins family. Several CTF-1 deletion mutants were tested for their ability to bind the viral origin as well as for their ability to activate DNA replication. We show that the DNA binding activity of CTF-1 is required for activation DNA replication. Moreover, gel shift experiments, suggest that AdPol and CTF-1 form a complex on the origin of DNA replication and that AdPol enhances the binding of CTF-1. On the basis of our observations and current available data, we propose a model for the initiation of Ad DNA replication.

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A STRATEGY FOR THE CLONING OF MAMALIAN DNA POLYMERASE δ (POL δ)

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Cloning and sequencing of the yeast *Saccharomyces cerevisiae* cdc2 gene, the functional homologue of pol δ of higher eukaryotes, revealed a much higher degree of similarity to herpesviral DNA polymerases than to other prokaryotic and eukaryotic replicative DNA polymerases. This allowed the definition of homology boxes that are present only in herpesviral DNA polymerases and in yeast pol δ (δ-like boxes). In order to clone pol δ from a mouse cDNA library we performed PCR using one primer corresponding to one of these boxes and another corresponding to a box highly conserved in all most replicative DNA polymerases. The PCR product had the length expected from the size of the homologous region in yeast pol δ. This DNA fragment was digested with *Sau*3A and subcloned. One of the subclones (170 bp) showed throughout its entire length 75% amino acid sequence identity to yeast pol δ. Besides its similarity to most replicative DNA polymerases this sequence is particularly homologous to δ-like boxes. We are now using this subclone for screening a NIH3T3 cDNA library to obtain the entire mouse pol δ cDNA.

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REGULATION OF RIBOSOMAL RNA SYNTHESIS IN SV40 INFECTED CELLS

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It has been reported that SV40 infection reactivates silent rRNA genes in human-mouse hybrid cells (Soprano et al., Mol. Cell. Biol., 3, 214, 1983). Recently, we developed a technique which allows to determine the proportion of active and inactive rRNA gene copies in a given cell type (Conconi et al., Cell, 57, 753, 1989). Therefore we decided to reinvestigate the behaviour of rRNA genes in SV40 infected cells. Up to 60 h post infection in TC7 cells, no significant changes in the proportion of active and inactive rRNA genes could be detected. This proportion also remained constant in mock infected cells as well as in COS cells (SV40 transformed cell line). Since rRNA synthesis increases upon infection (May et al., Exp. Cell. Res., 100, 433, 1976) this activation of transcription appears to be due to increased RNA polymerase I density or transcription rate and is unlikely to be due to activation of additional rRNA gene copies.

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DNA helicases from calf thymus

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Helicases are enzymes that transiently abolish the helical structure of the DNA to provide DNA and RNA polymerases with single stranded DNA. Starting from fetal and adult calf thymus tissue we have identified two DNA helicases. DNA helicase A has been purified to near homogeneity as a polypeptide of 47 kDa. This enzyme is dependent on the hydrolysis of ATP, dATP, CTP or dCTP and moves 3' -> 5' on the DNA strand it is bound to. Helicase B can be differentiated from helicase A by its elution behaviour on several chromatographic columns as well as by its nucleosidetriphosphate requirements. Research on the substrate preferences and processivities of the two helicases and on their interactions with other replicative enzymes are in progress. These include DNA polymerases α, δ and ε (see Weiser et al.) as well as the single stranded DNA binding protein known as replication factor A (see Georgaki et al.).

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Calf thymus replication factor A, an essential protein for DNA replication

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Replication factor A (RF-A) originally isolated from human cells is a heterotrimeric protein composed of polypeptides with Mr of 70, 32-34 and 11-14 kDa. It is a single-stranded (ss) DNA binding protein which is required for in vitro replication of simian virus 40 DNA. The ss-DNA-binding property is due to the 70 kDa subunit. RF-A is also essential for DNA replication in yeast and its 32-34 kDa subunit appears to be phosphorylated in a cell cycle dependent way.
RF-A was purified to apparent homogeneity by a several step procedure including chromatography on phosphocellulose, hydroxyapatite, DEAE-Sephacel, ss-DNA cellulose and Affigel Blue. The activity of RF-A was monitored by its binding to ssDNA using a filter binding assay. Similar as its human counterpart the purified calf thymus protein consists of polypeptides with Mr of 70, 55, 32 and 11 kDa. The 55 kDa subunit is likely a proteolytic breakdown product of the largest polypeptide. The identity of the calf thymus protein with RF-A was shown using a polyclonal antibody against the HeLa 32 kDa polypeptide (gift of T. Kelly), which also reacted in a immunoblot with the calf thymus 32 kDa subunit. Data on the stoichiometry of this protein with single-stranded DNA and its effect on the homologous three replicative DNA polymerases α, δ and ε will be shown.

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DNA POLYMERASES α, δ AND ε AND THEIR POSSIBLE ROLE AT THE EUKARYOTIC REPLICATION FORK

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Three different DNA polymerases have been identified so far that might have a functional role in nuclear DNA replication: DNA polymerases α, δ and ε. These three DNA polymerases can be isolated simultaneously from calf thymus, but the purification scheme has to be optimized for each of these enzymes. The two DNA polymerases containing a 3`- 5` exonuclease activity, DNA polymerases δ and ε were purified to apparent homogeneity by a four column procedure including DEAE-Sephacel, phenyl-Sepharose, phosphocellulose and hydroxyapatite. On hydroxyapatite DNA polymerase δ can completely be separated from DNA polymerase ε. Besides detailed biochemical comparison of the three DNA polymerases we present data on the processivity in the presence and absence of proliferating cell nuclear antigen (PCNA), a cell cycle regulated protein. Finally we introduce a new model on how the three DNA polymerase might act together at the eukaryotic replication fork.

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DNA POLYMERASE ACTIVITY IN THE CARBOXYTERMINAL PROTEOLYSIS PRODUCT p15 OF THE HIV 1 REVERSE TRANSCRIPTASE p66

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The HIV 1 pol gene product of 66 kDa (p66) is processed by the HIV inherent protease to the p66/51 reverse transcriptase/RNase H and a 15 kDa protein (p15) with a second RNase H activity. The p15 protein was found to have a DNA polymerase activity. Both enzymes were purified from an Escherichia coli expression vector by a three column procedure including DEAE-cellulose, phosphocellulose and heparin-Sepharose. DNA polymerase fractions containing the p66/51 heterodimer were devoid of the p15 protein and vice versa as shown with a monoclonal antibody directed against the carboxyterminal part of p66 and a polyclonal antibody directed against p66. Activity gel analysis demonstrated that the p15 protein revealed polymerizing activity. Processivity measurements and extensive templates studies showed that the p15 protein preferred RNA-templates and RNA primers, whereas the p66/51 heterodimer could in addition work on DNA templates with DNA primers. We therefore suggest that p15 might be required for the first step of HIV replication where the $tRNA^{lys}$ primed long terminal repeat is replicated to the 5´ end of the HIV genome.

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CASCADE REGULATION OF BOVINE HERPESVIRUS 1 IMMEDIATE-EARLY GENES

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Bovine herpesvirus 1 (BHV-1) contains two divergent immediate-early (IE) transcription units (Wirth, Vogt & Schwyzer, J.Virology 65, January 1991). Unit 1 encodes two alternative spliced IE RNAs, IER4.2 (4.2 kb) and IER2.9 (2.9 kb), with a common first exon. Unit 2 encodes one spliced transcript, IER1.7 (1.6-1.8 kb depending on strain). Northern blot, S1 and primer extension analysis showed that IER4.2 and IER2.9 were shut off in the absence of cycloheximide as regular IE genes, whereas the amount of IER1.7 steadily increased during lytic infection and was sensitive to inhibition of DNA synthesis. The promoter region of IER1.7 revealed 300 nt upstream from the transcription initiation site a tandem repeated sequence with high homology to other herpesviral origins of replication, which might explain the unexpected late kinetics. We also found an early transcript, ER2.6, with an initiation start site 20 nt upstream of exon 2 of IER2.9. This transcript was coterminal with exon 2 and displayed the same open reading frame. A minor IE transcript (IER6.3) was shown to contain sequences from both genome ends, implying that it arises from a circular genome (virion DNA is linear). Thus, the virus has evolved subtle mechanisms for temporal regulation of IE transcripts, which themselves encode regulatory proteins.

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GENOMIC FOOTPRINTING ANALYSIS OF THE VACCINIA VIRUS 7.5 kD GENE PROMOTER REGION

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Protein-DNA interactions in the 7.5 kD gene promoter region of vaccinia virus were studied by *in vivo* dimethylsulphate PCR and *in vitro* DNAase I footprinting techniques. Several footprints were detected, spanning over 130 bp (including the early and late phase vaccinia virus transcription start sites), indicating the involvement of cis-acting proteins. The footprints obtained by these methods with the different protein extracts and vaccinia virus DNA (representing the early and late phases of virus infection) may contribute to our present understanding of the regulation of early and late phase gene transcription.

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Tissue Tropism of Mouse Mammary Tumor Virus

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Epithelial cells of the mammary gland are not the only targets for the replication of Mouse Mammary Tumor Virus (MMTV) and several observations suggest that variants of the virus are involved in kidney and thymic cell transformation. The most obvious difference seen between MMTV-M (mammary origin) and MMTV-K (kidney origin) is located in the U3 region of the LTR, but mutations also occur in the structural genes of the viral genome, e.g. the *env* gene. Mice infected with MMTV-K harbor and express very low amounts of the virus detectable mainly by PCR and in situ hybridization. In contrast to the situation in mammary tumors caused by MMTV-M the kidney lesions of MMTV-K infected animals show little signs of viral replication. Epidemiological studies combined with the molecular analyses suggest that MMTV-K is not present in the germ line but horizontally transmitted by both males and females.
To study cell-specific gene expression several approaches have been chosen : 1. Transfection of both proviruses and of constructions of both LTR's with a reporter gene (HSV TK) into kidney and mammary cell lines. 2. In vitro transcription with various tissue and cell extracts. In parallel, DNA binding activities of these extracts have been tested. 3. Transfection of different cell lines with DNA constructions carrying different MMTV specific oligonucleotides in front of a heterologous promoter. And 4. Transgenic mice carrying the entire proviruses.

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REGULATORY INTERACTIONS IN THE PROMOTER REGION OF MOUSE MAMMARY TUMOR VIRUS DNA.

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To investigate factors that may cooperate with steroid receptors and modulate their action, we studied the binding in vitro of proteins from L-cell nuclear extracts onto the MMTV regulatory region. DNase I footprinting assays showed protection of the distal binding site for the glucocorticoid receptor, of its 5'-adjacent region, and of several sites in the basal promoter area. Alterations in the footprinting pattern were observed when the DNA contained mutations shown to affect the transcriptional activity in transfected cells. The specificity and the relative affinity of the interactions were analyzed by oligonucleotide competition experiments.

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MUTATIONS IN THE AUGs OF RSV LEADER ALTER TRANSLATION, BUT ALSO STRONGLY REDUCE VIRAL RNA PACKAGING

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RSV RNA leader sequence carries three open reading frames (uORF) upstream of the AUG initiator of the gag cistron. We have used site-directed mutagenesis to change two or three nucleotides in the four AUGs of RSV RNA leader (including the gag initiator) and to delete the first uORF. The five mutants were characterized in a transient assay for various parameters involving not only translation, but transcription, splicing and RNA packaging. Our results indicate that mutations of upstream AUGs or deletion of the first uORF all affected translation but to a different extent. Surprisingly however, these mutations had a dramatic effect on viral RNA packaging and reduced the infectivity of the progeny particles. Substitution of only two nucleotides in the first uORF was sufficient to lower viral RNA packaging by two orders of magnitude.

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FUNCTIONAL STUDIES ON THE ONCOMODULIN PROMOTER AND CLOSELY RELATED INTRACISTERNAL A PARTICLE (IAP) LTRs OF THE RAT

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Intracisternal A particles (IAPs) are endogenous, non-infectious retroviruses. Their proviral genomes are present in approximately 1000 copies in the DNA of rodents. A long terminal repeat (LTR) related to those of IAPs is inserted by transposition upstream to the oncomodulin gene. Oncomodulin is a low molecular weight Ca^{2+}-binding protein, first found in rat liver tumors. Oncomodulin is expressed in various rodent and human tumors and in the cytotrophoblasts of the rat and human placenta. We are interested in investigating promoter/enhancer activities as well as tissue specificities of the acquired oncomodulin LTR and of structurally related IAP LTRs of the rat. Therefore, we linked these LTRs upstream to a reporter gene (hGH) to carry out transient transfections in chemically transformed rat fibroblast cell lines. We used one cell line (T14c) known to synthesize high levels of endogenous oncomodulin and other cell lines of the same origin exhibiting little (T10) or no (T43) oncomodulin expression. First results indicate a distinct downregulation of IAP LTR fusion constructs in T43 cells as compared to T14 cells in relation to an inducible control promoter.

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ELECTRON MICROSCOPICAL 3D MODELING OF IMMUNOLABELED HIV-INFECTED CELLS

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Computer Aided Volumic Ultra Microscopy (CAVUM) corrects the artifacts caused by serial sectioning and reconstructs automatically the third dimension of the cells with the original normalized grey level information. The automatic extraction of cell borders allows to link the image analysis and treatments with image synthesis methods with minimal human intervention. The surface representation and the registered images provide an ultrastructural data base from which quantitative morphological parameters, as well as otherwise impossible visualizations, can be computed. This method takes advantages of correlative photon to electron microscopy and allows the reconstruction and morphometrical measurements of entire cells with 40 nm spatial resolution. Gold and/or peroxydase anti p24 immuno-labeling permits the precise selection of cultured HIV-infected cells for 3D morphometrical characterization and tentative labeling semi-quantification. These studies pave the way toward the use of single cells as reagent tubes and will be presented by computer-controlled projection.

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ANTIVIRAL POTENTIALS OF MUTANT FORMS OF HUMAN AND MURINE Mx PROTEINS

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Of the IFN-induced Mx proteins characterized to date, murine Mx1 and human MxA were shown to possess intrinsic antiviral activities. Mx1 and MxA proteins accumulate in different subcellular compartments: Mx1 is located in the nucleus, whereas MxA is cytoplasmic. Constitutive expression of either Mx1 or MxA protein in transfected 3T3 cells conferred resistance to influenza virus. Cell lines expressing MxA also acquired resistance to vesicular stomatitis virus (VSV).
Mutations affecting the nuclear transport signal located near the carboxy terminus destroyed the antiviral potential of Mx1 protein, suggesting that Mx1 inhibits a multiplication step of influenza virus taking place in the cell nucleus. MxA variants with mutations affecting the GTP-binding consensus motif failed to confer resistance to either VSV or influenza virus, indicating that this conserved domain is indeed necessary for Mx protein function. Mutating a single amino acid near the carboxy terminus destroyed the anti VSV activity of MxA protein but did not affect the anti-influenza virus activity. None of the Mx protein hybrids that we tested possessed antiviral activity. Our data demonstrate that a functional analysis of Mx protein domain is feasible. This approach will help determining the modes of Mx protein action.

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MAPPING OF THE VACCINIA VIRUS "RELEASE GENE"

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Plaque formation in Vaccinia Virus is inhibited by the compound N_1-Isonicotinoyl-N_2-3-Methyl-4-Chloro-BenzoylHydrazine (IMCBH). We have isolated a mutant virus that forms wild-type plaques in the presence of the drug. Comparison of wild-type and mutant virus showed that both viruses produced similar amounts of infectious, intracellular naked virus in the presence of the drug. However, in contrast to the mutant, no extracellular enveloped virus was obtained from IMCBH treated cells infected with wild-type virus. Marker rescue experiments were used to map the mutation conferring IMCBH resistance to the mutant virus. The map position coincided with that of the previously identified gene encoding the viral envelope antigen of Mr 37000. Sequence analysis of both wild-type and mutant genes showed a single nucleotide change which in the deduced amino acid sequence changes an Asp in the wild-type gene to Tyr in the mutant.

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INTERFERENCE OF Mx PROTEINS AT DISTINCT STEPS OF THE INFLUENZA VIRUS AND VSV MULTIPLICATION CYCLE.

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Human MxA and murine Mx1 are closely related interferon-induced proteins with intrinsic antiviral activities. Constitutive expression of cDNAs encoding either Mx1 or MxA protein in transfected 3T3 cells generated an antiviral state against influenza virus. Surprisingly, cells expressing MxA protein also acquired resistance to vesicular stomatitis virus (VSV).
To identify the viral replication steps blocked by Mx proteins, we examined the accumulation of viral mRNAs and proteins following infection with influenza virus or VSV. Viral proteins did not accumulate to significant levels in Mx-transfected cells resistant to influenza virus or VSV infection. Accumulation of primary transcripts of influenza virus genes was strongly reduced in cells expressing Mx1, but was not affected in cells expressing MxA. However, the levels of VSV primary transcripts, in particular the L mRNA (encoding the VSV polymerase) were decreased in cells expressing MxA compared to control cells.
These results indicate that Mx1 inhibits the replication cycle of influenza virus at the step of primary transcription. In contrast, MxA blocks the multiplication of influenza virus at a replication step following primary transcription, but prior to or including protein synthesis. However, MxA appears to interfere with the primary transcription of VSV.

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Sendai virus can bud in absence of any detectable hemagglutinin-neuraminidase glycoprotein

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Sendai virus is an enveloped virus containing two surface glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (Fo) proteins, responsible for respectively attachment and penetration of the virus particle in the host cell. The virus particle is formed by insertion of these viral glycoproteins in the infected cell plasma membrane, by organization of the matrix (M) protein as a leaflet structure under the plasma membrane, by recognition of this modified plasma membrane by the nucleocapsid and by formation of a bud. A temperature sensitive mutant in HN (SVts271) is known to efficiently bud at non-permissive temperature in absence of any detectable HN, suggesting that presence of the major viral glycoprotein is not required for correct budding. The presence of the transmembrane and cytoplasmic portions (TCP) of HN has nevertheless never been excluded. In the present study, it is shown that no HN-TCP can be found in ts271 particles produced at 39°C, in conditions where a TCP is identified after shaving the wild type virus particles with bromelain. This excludes the quantitative participation of HN in the process of viral budding and lowers the minimal requirements for viral particle assembly down to one glycoprotein (Fo), M and the viral nucleocapsid.

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TOWARDS MEASLES VIRUS-BASED EXPRESSION VECTORS

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We described a procedure for generating infectious measles virus (MV) from a DNA copy (15.894 base pairs) of the MV RNA genome (EMBO J. 9 (1990) 379-384). Modified versions of the full length MV cDNA in which strategic restriction sites have been eliminated or added were found to retain infectivity. Subcloning vectors allowing easy exchange of mutated genes and their transfer in full-length vectors are also available now. Using this system, the functions of potentially defective MV genes, and of the editing signal, are being analysed (posters by Ballart, Schmid, and Kälin). Moreover, the reading frame potentially encoding a 328 amino acid long amino-terminal extension of the fusion protein has been interrupted, but the corresponding cDNA was not found to loose infectivity. To analyse the process of viral budding, the intracellular domains of the viral envelope proteins are being exchanged with those of related viruses. We have also introduced in MV full length cDNAs the framework for a seventh MV transcription unit, from which potentially additional proteins might be expressed.

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SPECIFIC ALTERATION OF THE MEASLES VIRUS FUSION PROTEIN CYTOPLASMIC DOMAIN AS A PREREQUISITE FOR PATHOGENIC PERSISTENCE?

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In all three morbilliviruses analyzed the last 14 of the 33 amino acid long cytoplasmic domain of the fusion (F) protein, which is thought to interact specifically with the morphogenic matrix protein, are strictly conserved. Conversely, in the 11 analysed cases of SSPE (for definition see abstract by Ballart et al.) drastic alterations were monitored in a majority of the individual clones corresponding to the relevant F gene region. Only from three cases clones corresponding to the wild type consensus sequence were isolated in addition to clones containing mutated sequences. Mutations included four different premature terminations, one elongation beyond the normal stop codon, one frameshift, and missense mutations involving six, four or one amino acid replacements. Representative mutated F gene segments are now inserted into genomic "infectious" cDNA to test the altered F proteins for competence in virion formation.

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INTRACELLULAR LOCALIZATION OF MEASLES VIRUS PROTEINS

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The functional characterization of variant measles virus (MV) genes, e.g. from brain autopsies of victims of MV-induced syndromes, requires expression in eukaryotic cells. Initial efforts based on lipofection of synthetic capped and polyadenylated MV RNAs failed, in spite of positive results obtained with similar RNA encoding chloramphenicol acetyl transferase. Efficient transient expression of the MV nucleocapsid (N), phosphoprotein (P) and hemagglutinin protein was recently obtained in *cos* cells from a vector based on a cytomegalovirus promoter and a SV40 origin of replication. N protein expressed alone was localized largely in the nucleus. P protein, in contrast, showed immunostaining exclusively at fibrillar structures in the cytoplasm. Coexpression of N and P protein resulted in localization of the N protein both at the nucleus and fibrillar structures in the cytoplasm. To study the transport and interactions of MV proteins derived from lytic or persistent viruses, and to allow complementation studies, we are currently constructing vectors allowing expression of all MV proteins.

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FUNCTIONAL AND NON-FUNCTIONAL MEASLES VIRUS MATRIX GENES FROM LETHAL HUMAN BRAIN INFECTIONS

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Subacute sclerosing panencephalitis (SSPE) is a lethal disease induced by the persistence of measles virus in the human brain. In many SSPE cases, the viral matrix (M) protein cannot be detected; in others, M proteins of the expected size are found and the sequence analysis of M cDNAs has not shown striking alterations. To test whether the functional inactivation of the MV M gene is always linked to disease development, we have replaced the M gene of infectious full length genomic cDNA (EMBO J. 9 (1990) 379-384) with different M genes derived from four SSPE cases. One of the SSPE M genes tested proved to be functionally competent, giving rise to a virus yielding titres similar to those of viruses containing the M gene from a control lytic strain. In the other three SSPE cases analyzed, infectious virus could be recovered by exchanging the amino-terminal half of the M protein with the corresponding Edmonston gene fragment, but not by exchanging the whole M protein or only the carboxyl-terminal half.

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ROLE OF AP-1 IN THE CELL-TYPE SPECIFIC EXPRESSION OF HUMAN PAPILLOMAVIRUS 18 GENES.

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The long control region of human papillomavirus 18 (HPV-18) DNA has two binding sites for the AP-1 jun/fos family of proteins, one proximal to the P104 promoter and the other upstream in the enhancer. They are involved in controlling HPV-18 transcription. We have shown that the tumour promoter TPA stimulates the activity of proteins binding to both sites. TPA also increases the level of mRNA synthesized from integrated HPV-18 DNA in HeLa cells. HPV-18 is cell-type specific and believed to replicate in keratinocytes. The major complex formed with oligonucleotides containing either AP-1 site and proteins from HaCaT keratinocytes or HeLa cells is not detected with proteins from several types of low passage human fibroblasts. This may account, in part, for the cell specificity of the virus. The absence of the major AP-1 activity from fibroblasts is not due to the absence of fos mRNA. With longer fragments of DNA from the enhancer (but not the promoter) and fibroblast proteins, some AP-1 binding is detected. Since the AP-1 core sequence is the same in the two sites, this binding may involve flanking DNA sequences or cooperation with other proteins.

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INHIBITION OF INFLUENZA VIRUS INFECTION BY A MONOCLONAL ANTIBODY AGAINST ENDO-LYSOSOMAL PROTEIN.

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A monoclonal antibody recognizing 120kD protein in lysosome was obtained using a human osteosarcoma cell as an antigen. The antibody, named OSW2, stained the cell surface very weakly and more than 80% of the antigen localized inside the cell. The surface bound OSW2 was rapidly endocytosed and some of them recycled but most of them were slowly transported to lysosomes. We have found both the whole IgG and the Fab fragment of OSW2 inhibited influenza virus infection at less than 10μg/ml. OSW2 did not inhibit the hem-agglutination of the virus. Acid-induced confor-mational change of HA in endosomes was found to be reduced by OSW2. In contrast, OSW2 did not inhibited the infection of sendai virus. These results suggest OSW2 inhibited some animal virus infection by acting on the endocytic compartments.

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INFLUENCE OF THE OCTADECYLRHODAMINE B CHLORIDE (R18) MEMBRANE MARKER ON THE ACTIVITIES OF PR8 INFLUENZA VIRUSES

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Octadecylrhodamine B chloride has been introduced by Hoekstra et al. (*Biochemistry 23*, 5675, 1984) as a marker to quantitate membrane interactions. R18 is integrated into membranes either upon formation of lipid bilayers (i.e. liposomes) or by external addition to biological membranes (i.e. viruses). Interaction of labeled with unlabeled membranes leads to dequenching, i.e. an increase in fluorescence. Using R18-labeled liposomes, two types of interactions between virus receptor (GD1a)-containing liposomes and PR8 viruses could be distinguished at 37°C: a fast hemagglutinin-specific fusion at pH5.3 and a slow, nonspecific lipid transfer at pH7.4 (Wunderli and Ott, *Biochemistry 29*, 1990, 1990). In order to study the effect of R18 incorporation into biological membranes, PR8 viruses were labeled and tested for their biological activities. The rate constant for fusion was significantly lower in these incubations as compared to those with R18-labeled liposomes indicating that R18-labeling leads to partial inactivation of the virus. This finding is strongly supported by the fact that other viral activities like infectivity, hemolysis and hemagglutination of R18-labeled viruses are also reduced.

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THE ELIMINATION OF MOUSE HEPATITIS VIRUS BY TRANSITIONAL TRANSPLANTATION OF HUMAN TUMOS FROM INFECTED ATHYMIC MICE INTO AHTYMIC RATS

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A nude mouse colony contained in an isolated unit was found to harbour MHV despite the fact that all hygienic precautions were taken. The virus spread rapidly causing a high mortality rate predominantly in experimentally used animals. Moreover, we observed a high percentage of tumor regression in our tumor transplanted mice. Attempts to eliminate the MHV by repeated tumor transplantation into virus-free nude mice were unseccessful.
Since MHV has a limited host range, we transplanted, in parallel, four different lines of human nephroblastomas as solid tumors from athymic mice in athymic rats and parts of the same tumors in "fresh" nude mice. All manipulations were performed in isolators. Detection of MHV was done twice by a serological check of six week old sentinels.
The results showed transmission of MHV-infection in the control mice under gnotobiotic conditions as previously found in the normal animal room. On the other hand no evidence of infection was detected in the transplanted nude rats and also after retransplantation to nude mice.
We hypothesize that the virus is harboured in the stroma cells of the murine host but not in the rat host nor in the human tumor cells. Histological comparision showed no alteration of specific tumor morphology in different hosts.

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GENETICALLY ENGINEERED POTATO VIRUS Y^N-RESISTANCE IN TOBACCO AND POTATO.

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The Potato Virus Y (PVY) is the type member of the largest and most devastating group of plant viruses. We obtained resistance to PVY necrotic strain 605 (PVY^N 605) in SR1 tobacco transformed with PVY^N 605 coat protein (CP) gene cloned either in its sense or its antisense orientation. Using homozygous R_2 progeny we show that the CP-mediated resistance is also effective against PVY common strain O, and provides tolerance against Potato Virus V (PVV). However, antisense-mediated resistance/tolerance is restricted to PVY^N 605. Mixed PVY^O and PVV inoculation of transgenic tobacco containing the CP gene in its sense orientation shows that PVV development does not reduce resistance to PVY^O.
We also obtained Bintje potato transformed with PVY^N 605 CP gene totally resistant to this virus. We are conducting experiments to determine the range of this protection against different potyviruses.
We present evidences that the CP is necessary for protection against PVY^N 605 in tobacco, by using a construction in which a stop codon was inserted at the beginning of the CP gene.
In order to understand the mechanisms involved in the CP-mediated resistance we performed different grafting experiments.

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QUANTITATIVE ESTIMATION OF SEMLIKI FOREST VIRUS (SFV) CAPSID (C) PROTEIN DURING NUCLEOLAR ACCUMULATION

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By scanning western blots developed with affinity purified anti-C protein antibody we quantified C protein accumulation into nucleoli of SFV infected cells. We could show that a minor part of C protein migrates from the cytoplasm through the nucleoplasm to the nucleolus both in vertebrate and invertebrate cells. Using high concentrations of cycloheximide to block translation, karyophilic C protein was shown to be parental. Migration is a rapid event being completed within 1.5 hpi in CV-1 and Aedes albopictus cells. Increase of the amount of nucleolar C protein during the viral replication in CV-1 cells suggests that also progeny (newly synthesized) C protein accumulates into nucleoli.

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Mutational analysis of a bacteriophage P1 late promoter sequence.

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Bacteriophage P1 late promoter sequences control the expression of either morphogenic or late regulatory functions and are only transcriptionally active during the second half of a lytic growth cycle. The late promoter sequences found, are highly homologous, and differ from an *E. coli* consensus promoter in as much as they contain a -10 Pribnow box, but lack any homology to the -35 region. We investigated the promoter of the P1 tail fibre operon, called P_S, in search for sequences which are essential for late promoter activity. Degenerated oligonucleotides, carrying randomly distributed single basepair changes, were used as primers in an *in vitro* oligonucleotide directed mutagenesis reaction. This allowed us to isolate a set of 57 single point mutations, which were then tested for promoter activity. A region between position -31 and -18, as well as the -10 box proved to be important. It can be postulated that a 9 bp inverted repeat located within the former region might serve as a binding site for the single phage encoded function, which is required for late promoter activity during lytic growth.

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TWO-DIMENSIONAL DNA-FINGERPRINTING IN ANIMALS

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We have optimized the DNA-fingerprinting method for pigs, dogs and mouse inbred strains. The method is a powerful technique to identify humans or animals, but it has some limitations for genetic linkage analysis. The resolution of an agarose gel is too low to separate all minisatellites in a single lane. In addition, by using the Jeffreys core probes 33.15 and 33.6 in pigs and dogs, fewer polymorphic bands were detected compared to humans. We therefore began to analyze animal genomes with the two-dimensional DNA-fingerprinting method, which was recently introduced in humans (Uitterlinden et al., Proc.natl.Acad.Sci. USA, 86, 2742-2746, 1989). This technique had to be adapted for these three species (Schelling et al., in:DNA-fingerprinting, T.Burke,A.J. Jeffreys, R. Wolff, G. Dolf eds., Birkhäuser Verlag, Basel, in press,1991) and we will discuss the advantage of this technique and the gain of information content compared to the generally used one-dimensional one.